ABSTRACT

MUTATIONAL STUDIES IN THE dNTP BINDING POCKET OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REVERSE TRANSCRIPTASE

by
Smita Shukla

It was earlier postulated that Gln 91 of HIV-1 RT stabilizes the side chain of Tyr 183 via hydrogen bonding interaction between O(H) of Y183 and CO of Q91 (Harris et al., BIOCHEMISTRY 37: 9630, 1998). In an attempt to understand the function of Gln 91 in the catalytic mechanism, mutants of this residue (Gln91→Ala and Gln91→Asn) were generated and subjected to an in-depth analysis. The efficiency of reverse transcription of natural U5-PBS HIV-1 RNA template was severely impaired by both conservative and non-conservative substitutions from Gln→Asn and Gln→Ala, a result similar to that observed with Y→F substitution at position 183. The major defect seems to be at the substrate binding step as the processivity of these mutants was not affected while the extent of primer utilization correlated with the loss of their polymerase activity. Curiously, these mutant derivatives of Q91 were found to be highly resistant to ddNTPs and exhibited greater stringency in discriminating between correct and incorrect nucleotides. These results suggest possible interaction of Q91 with other residues in the dNTP binding pocket that may be responsible for conferring greater flexibility to the pocket. A model is proposed which suggests that subtle structural changes due to mutation in the region may have influenced the active site of the enzyme that may interfere in the substrate recognition.

MUTATIONAL STUDIES IN THE dNTP BINDING POCKET OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REVERSE TRANSCRIPTASE

by
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To my beloved mother

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ABBREVIATIONS

A (ala)	Alanine
Q (gln)	Glutamine
M (met)	Methionine
N (asn)	Asparagine
R (arg)	Arginine
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
DTT	Dithiothreitol
PMSF	Phenylmethylsulfonylfluoride
IPTG	Isopropyl-beta-thiogalactopyranoside
Poly (rA). $(dT)_{18}$	Polyriboadenylic acid annealed with -(Oligodeoxythymidylic acid)18
dNTP	DeoxyriboNucleoside Triphosphate
dATP	DeoxyAdenosine Triphosphate
dGTP	DeoxyGuanosine Triphosphate
dCTP	DeoxyCytosine Triphosphate
dTTP	DeoxyThymidine Triphosphate
ddNTP	Dideoxyribonucleoside Triphosphate
HIV-1 RT	Human Immunodeficiency Virus type 1 Reverse Transcriptase
IMAC	Immobilized Metal Affinity Chromatography
IDA-Sepharose	Iminodiacetic acid-Sepharose

MuLV	Murine Leukemia Virus
PPi	Pyrophosphate
PFA	Phosphonoformic Acid
U5-PBS HIV-1 RNA template	HIV-1 genomic RNA template corresponding to Primer binding sequence region
U5-PBS HIV-1 DNA template	DNA template corresponding to the U5-PBS HIV-1 genomic RNA sequence

CHAPTER 1

INTRODUCTION

This thesis pertains to investigations on structure and function of reverse transcriptase (RT) of human immunodeficiency virus (HIV), the retrovirus known to be the causative agent of acquired immune deficiency syndrome, viz. AIDS. This clinical syndrome, characterized by a marked reduction in the number of CD4+ cells along with the development of opportunistic infections (sometimes leading to cancer e.g. Kaposi's Sarcoma), results from persistent replication and spread of HIV (Levy, 1998). In the life cycle of HIV in human cells, RT plays a critical role in the synthesis of double-stranded pro-viral DNA, which then gets integrated into the host human cell DNA (Coffin, 1999).

Ever since the discovery of HIV in 1983, science has responded to the challenge of AIDS by rapidly identifying etiology, describing pathogenesis and transmission routes, and developing diagnostic tests and treatment. However, this did not prevent the global spread of HIV, with over 25 million fatal cases so far, another 33 million infected, and disastrous socioeconomic and demographic consequences. To meet the goal of universal access to prevention, treatment and care by 2010 would require a quadrupling of funding to an estimated US$42 billion by 2010, including adequate overall strengthening of healthcare systems (Kallings, 2008).

1.1 Structure of the Virion

1.1.1 Visualization of HIV Structure

The morphology and overall arrangement of HIV particles have been characterized by a host of electron microscopic observations (in thin sections), However, the visualization of mature and immature HIV virions by cryo-electron microscopy (which maintains the sample in a thin layer of vitrified water) has been successful to some extent (Coffin, 1992; Vogt, 1997). HIV particles appear to be roughly spherical, membrane-bound structures, with diameter between 1000-3000A^{o}. These include budding membrane-bound virus-like particles (immature virions) and mature particles. The **panoply** of shapes and sizes of particles in a preparation (for electron microscopic examination) demonstrates that particle formation does not require the isomeric structure and that the assembling of HIV particles is an enormously robust process. As Wilk and Fuller (1999) stated in their excellent review, "The virus appears to be less concerned about the icosahedral symmetry than the structural biologist." Notwithstanding this apparent setback in the structural studies of HIV, recent findings have expanded our knowledge of its major structural proteins. Further challenge lies in understanding the changes in structure and the interactions of these components during assembly and maturation.

1.1.2 Overall Structural Features of HIV (McClure et al., 1988; Levy, 1998)

HIV is a member of the Lentivirus genus, which includes retroviruses having complex genomes and cone-shaped capsid core particles. The general features of the mature HIV virion and known or postulated localization of mature proteins are schematically depicted in Fig.1.1 (McClure et al., 1988). All lentiviruses are enveloped by a lipid bilayer which seems to be derived from the host cell membrane. Exposed surface glycoproteins (SU;

gp120) are anchored to the virion via interactions with the transmembrane protein (TM; gp41). The lipid layer also contains quite a few host cell membrane proteins, such as: major histocompatibility antigens, actin, and ubiquitin. A matrix shell possessing about 2000 copies of the matrix protein (MA; p17) lines the inner surface of the viral membrane.

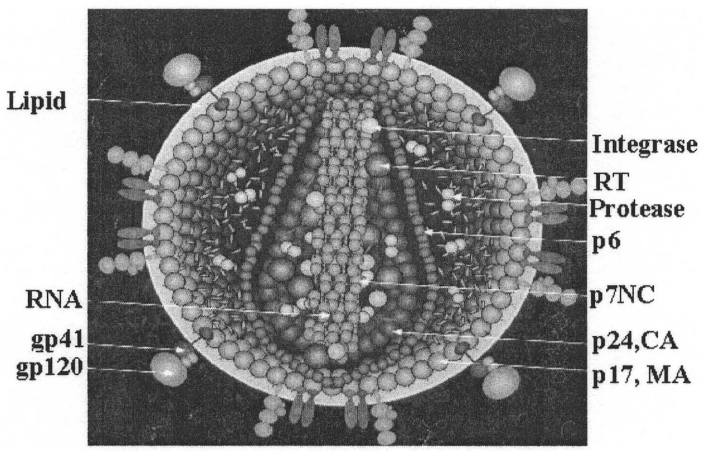

Figure 1.1 Structure of HIV-1 Virion Particle.

At the center of the virion, a core particle composed of some 2000 copies of the Capsid protein (CA; p24) is localized. This capsid particle also clusters two copies of the viral RNA genome, which are stabilized as a ribonucleoprotein complex with approximately 2000 copies of the nucleocapsid protein (NC; p7). The core particle also houses three essential virally encoded enzymes: Protease (PR), reverse transcriptase (RT) and integrase (IN). The virion encloses the accessory proteins: Nef, Vif and Vpr. Three additional virally encoded accessory proteins viz., Rev, Tat and Vpu, which provide essential regulatory functions in the host cell, do not appear to be packaged in the virion.

1.2 HIV Replication Cycle

Mature HIV-1 particles begin their life-cycle by recognition of the target cells, viz., the cells bearing CD4+, a protein that normally functions in immune recognition. The early phase of the life cycle begins with CD4+ recognition and proceeds step-wise to the formation, and later, to the integration of the pro-viral DNA into the host cellular DNA. The late phase comprises the events from the transcription of the integrated pro-viral genome to the virus budding and maturation.

1.2.1 Entry to the Cell

HIV enters macrophages and $CD4^+$ T cells by the adsorption of glycoproteins on its surface to receptors on the target cell followed by fusion of the viral envelope with the cell membrane and the release of the HIV capsid into the cell (Chan et al., 1998; Wyatt et al., 1998). Entry to the cell begins through interaction of the trimeric envelope complex (gp160 spike) and both CD4 and a chemokine receptor (generally either CCR5 or CXCR4, but others are known to interact) on the cell surface (Chan et al., 1998; Wyatt et al., 1998). gp120 binds to integrin $\alpha_4\beta_7$ activating LFA-1 the central integrin involved in the establishment of virological synapses, which facilitate efficient cell-to-cell spreading of HIV-1 (Arthos et al., 2008) The gp160 spike contains binding domains for both CD4 and chemokine receptors (Chan et al., 1998; Wyatt et al., 1998). The first step in fusion involves the high-affinity attachment of the CD4 binding domains of gp120 to CD4. Once gp120 is bound with the CD4 protein, the envelope complex undergoes a structural change, exposing the chemokine binding domains of gp120 and allowing them to interact with the target chemokine receptor (Chan et al., 1998; Wyatt et al., 1998). This allows for a more stable two-pronged attachment, which allows the N-terminal fusion peptide gp41

to penetrate the cell membrane (Chan et al., 1998; Wyatt et al., 1998). Repeat sequences in gp41, HR1 and HR2 then interact, causing the collapse of the extracellular portion of gp41 into a hairpin. This loop structure brings the virus and cell membranes close together, allowing fusion of the membranes and subsequent entry of the viral capsid (Chan et al., 1998; Wyatt et al., 1998).

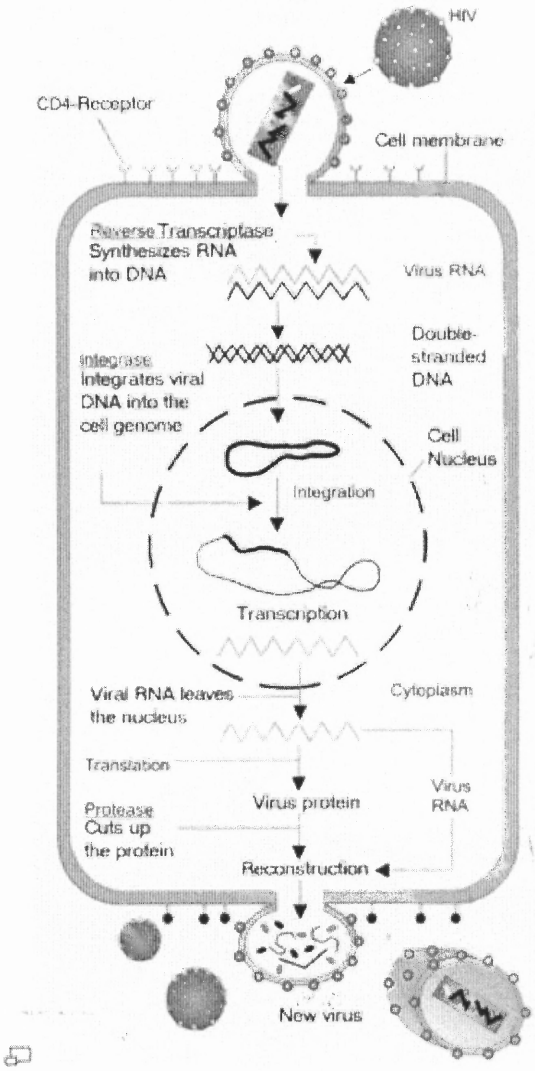

Figure 1.2 The HIV Replication Cycle.

Once HIV has bound to the target cell, the HIV RNA and various enzymes, including reverse transcriptase, integrase, ribonuclease and protease, are injected into the cell (Chan et al., 1998). During the microtubule based transport to the nucleus, the viral single strand RNA genome is transcribed into double strand DNA, which is then integrated into a host chromosome.

1.2.2 Replication and Transcription

Once the viral capsid enters the cell, an enzyme called reverse transcriptase liberates the single-stranded (+)RNA from the attached viral proteins and copies it into a complementary DNA (Zheng et al., 2005). This process of reverse transcription is extremely error-prone and it is during this step that mutations may occur. Such mutations may cause drug resistance. The reverse transcriptase then makes a complementary DNA strand to form a double-stranded viral DNA intermediate (vDNA). This vDNA is then transported into the cell nucleus. The integration of the viral DNA into the host cell's genome is carried out by another viral enzyme called integrase (Zheng et al., 2005)

This integrated viral DNA may then lie dormant, in the latent stage of HIV infection (Zheng et al., 2005). To actively produce the virus, certain cellular transcription factors need to be present, the most important of which is NF-κB (NF kappa B), which is upregulated when T cells become activated (Hiscott et al. 2001). This means that those cells most likely to be killed by HIV are those currently fighting infection.

In this replication process, the integrated provirus is copied to mRNA which is then spliced into smaller pieces. These small pieces produce the regulatory proteins Tat (which encourages new virus production) and Rev. As Rev accumulates it gradually starts to inhibit mRNA splicing (Pollard et al., 1998). At this stage, the structural proteins

Gag and Env are produced from the full-length mRNA. The full-length RNA is actually the virus genome; it binds to the Gag protein and is packaged into new virus particles.

HIV-1 and HIV-2 appear to package their RNA differently; HIV-1 will bind to any appropriate RNA whereas HIV-2 will preferentially bind to the mRNA which was used to create the Gag protein itself. This may mean that HIV-1 is better able to mutate (HIV-1 infection progresses to AIDS faster than HIV-2 infection and is responsible for the majority of global infections).

1.2.3 Assembly and Release

The final step of the viral cycle, assembly of new HIV-1 virons, begins at the plasma membrane of the host cell. The Env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by protease and processed into the two HIV envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. The Gag (p55) and Gag-Pol (p160) polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. Maturation either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion (Gelderblom, 1997). This cleavage step can be inhibited by protease inhibitors. The mature virus is then able to infect another cell.

1.3 Reverse Transcriptase

In retroviruses such as the human immunodeficiency virus type 1 (HIV-1), reverse transcriptase (RT) is the sole enzyme necessary for the catalytic transformation of single-stranded viral RNA into the double-stranded linear DNA that is integrated into host cell chromosomes. HIV-1 RT is composed of two subunits of 66 kDa and 51 kDa (p66 and p51). The N-terminal 440 amino acids of p66 constitute the polymerase domain and the C-terminal 120 amino acids comprise the RNase H domain. The p51 subunit of HIV-1 RT corresponds to the polymerase domain of the p66 subunit (Jacobo Molina., 1993). The polymerase domain of the p66 subunit is folded into an open conformation containing the polymerase active site cleft while the inert p51 subunit is closed and compact (Wang et al., 1994). The polymerase cleft resembles open right hand and folds into fingers, palm and thumb sub-domains.

1.4 Drugs Targeting RT

Considerable effort has been expended in discovering compounds that inhibit HIV-1 RT, an essential viral enzyme which is responsible for the conversion of the (+) single-stranded RNA viral genome into double-stranded DNA (dsDNA), which subsequently becomes integrated into host cell chromosomes. RT is an attractive target of drug therapy not only because it is essential for HIV replication, but also it is not required for normal host cell metabolism.

HIV-1 RT inhibitors can be divided into two classes (Larder, 1993): nucleoside analogs (also referred to as nucleoside inhibitors, chain terminators, or competitive inhibitors) and non-nucleoside inhibitors (also refereed to as TIBO-like inhibitors). Many of the nucleoside analogs (Balzarini et al., 1999) cause termination of the growing DNA

chain. Because they closely resemble normal nucleosides, nucleoside analog inhibitors can be added to the newly synthesized DNA during reverse transcription. Elongation is blocked because the chain terminators lack the 3'-OH functional group essential for incorporation of additional nucleotide. All of the currently marketed AIDS drugs are of this class. Although these drugs are selective for HIV-1RT, they are not highly specific and can inhibit normal cellular polymerases, causing serious side effects.

The non-nucleoside inhibitors do not function as chain terminators, and do not bind at the dNTP-binding site. These compounds are non-competitive inhibitors with respect to dNTP. Several families of such compounds have been reported, including TIBO (Pauwels et al., 1990), dipyridodiazepinone, pyridinone, BHAP, HEPT, α-APA and inophyllum derivatives (reviewed, Tantillo et al., 1994). Although these inhibitors exhibit striking structural differences and fall into several chemical groups, they all contain aromatic moieties and bind to the same hydrophobic pocket in HIV-1RT.

1.5 Drug Resistance

Although there has been substantial progress in blocking HIV-1 replication in vitro, the success of most in vitro experiments and numerous clinical trials to date has been limited due to the emergence of viral strains that have point mutations in the region encoding HIV-1RT and prevent these drugs from inhibiting RT. Mutations that render HIV-1RT drug-resistant have been encountered with every RT inhibitor so far tested. Sequence analysis has identified the point mutations responsible for resistance but a great deal still remains to be known concerning the molecular basis of resistance mechanisms. The structure determination of HIV-1RT with bound template-primer reported by Jacobo-

Molina et.al., in 1993 (Jacobo-Molina et al., 1993), provides the location of HIV-1RT inhibitor-resistance mutations in a three-dimensional context, which makes it possible to formulate hypotheses for the mechanisms of drug resistance that are based on the structure and the location of the specific mutations that lead to drug resistance.

1.6 Background of this Project

The rapid emergence of Human Immunodeficiency Virus (HIV-1) strains resistant to specific inhibitors has frustrated all efforts to control the spread of acquired immunodeficiency syndrome. Dynamics of HIV-1 replication *in vivo* have demonstrated that within 2-4 weeks of treatment with nucleoside analogs, the wild type virus in plasma is completely replaced by the drug resistant mutants (Wei et al., 1995; Ho et al., 1995).

The single stranded HIV-1 viral RNA genome is efficiently converted into the double stranded proviral DNA by the virally encoded reverse transcriptase, which is essential for viral replication and establishing infection. The functional form of mature HIV-1 RT contains two subunits, p66 and p51; the smaller subunit is derived from the proteolytic cleavage of the larger subunit (di Marzo-Veronese et al., 1986; Lightfoote et al., 1996). A number of high resolution crystal structure of HIV-1 RT in apocrystal form, liganded with inhibitors, bound with DNA as well as the most elegantly solved structure of enzyme-DNA-dNTP ternary complex are now available. In the crystal structure, the polymerase domain of the larger subunit is folded into an open conformation containing the polymerase active site cleft while the inert smaller subunit is closed and compact (Wang et al., 1994). The polymerase cleft resembles open right hand and folds into fingers, palm and thumb sub-domains. A large number of amino acid

residues in the polymerase domain of this enzyme have been subjected to extensive mutagenesis. However, only a few of them have been subjected to in-depth biochemical characterization in order to understand their functional role in the catalysis. The mutant derivatives of amino acid residues at positions 65, 72, 89, 110, 115, 151, 160, 183, 184, 185, 186, 219, 249, 307, 311 and most of the residues in Motif- E (residues 227-237) and in the thumb sub domain (residues 253-271) have been extensively characterized and their functional roles in the context of three-D structure have been proposed.

Earlier the mutant derivatives of catalytically important residues of YMDD motif have been extensively analyzed and their functional roles in the 3-D context have been proposed. One of the residues in this motif that has been studied in detail is tyrosine at position 183 (Harris et al., 1998). A conservative substitution of Tyr183 to Phe183 resulted in 70% loss of polymerase activity with a significant increase in the fidelity of DNA synthesis. Based on experimental results and molecular modeling studies, it was predicted that the side chain of Y183 is stabilized via hydrogen bonding interaction with the side chain of Gln 91 (Harris et al., 1998). This interaction was indeed observed in a recently solved 3-D crystal structure of RT-DNA-dNTP ternary complex (Huang et al., 1998). The loss of polymerase activity by Tyr → Phe substitution may have manifested due to loss of this hydrogen bonding interaction. Thus, similar influence on the polymerase function of the enzyme may be expected if this hydrogen bonding interaction is perturbed by mutation at position 91. This work reports here that the removal or reduction in the length of the side chain at position 91 (Gln → Ala or Gln → Asn) resulted in significant loss of polymerase activity of the enzyme probably due to loss of

hydrogen bonding interaction with Tyr183. These results have been discussed in the context of the recently solved three dimensional crystal structure of HIV-1 RT-DNA-dNTP ternary complex (Huang et al., 1998).

CHAPTER 2

MATERIALS AND METHODS

Mutagen-M13 in vitro mutagenesis kit was obtained from Bio-Rad laboratories while Sequenase and DNA sequencing reagents were from U.S. Biochemicals. Restriction endonucleases, DNA-modifying enzymes and HPLC-purified dNTPs were from Boehringer Mannheim or Promega; IDA-Sepharose for immobilized metal affinity chromatography (IMAC) was purchased from Pharmacia and ^{32}P-labeled dNTPs and ATP were obtained from DuPont / New England Nuclear Corp. Synthetic template-primers, sequencing primers and mutagenic oligonucleotides were synthesized at the Molecular Resource Facility at UMDNJ. An HIV-1 RNA expression clone pHIV-PBS was a generous gift from Dr. M.A. Wainberg (Lee et al., 1998). All other reagents were of the highest available purity grade and were purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad.

2.1 Expression of Plasmid Clones and In Vitro Mutagenesis

Two recombinant plasmids, pKK-RT66 and pET-28a-RT51 containing P66 and P51 encoding regions were used for isolating wild type heterodimeric HIV-1 RT (Pandey et al., 1996). The smaller subunit contained His-tag sequences at the N-terminal region. The EcoR I and Kpn1 fragment (1.432 kb) of pKK-RT$_{66}$ encoding the polymerase domain of HIV-1 RT was sub cloned in bacteriophage M13 mp18 and used as the template for site directed mutagenesis. The mutagenesis protocol-using dU 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: The EcoRI and Kpn1 fragment from M13 mp18 was cloned in RT_{66} expression cassette and the Bal1 and Kpn I fragment was cloned in RT51 expression cassette.

2.2 Polymerase Activity Assay

An HIV-RNA expression clone (pHIV-PBS) was used for the preparation of U5-PBS HIV-1 genomic RNA template as described earlier (Lee et al., 1998). Polymerase activity of the WT and mutant enzymes was assayed on U5-PBS HIV-1 RNA and 49 mer U5-PBS DNA templates primed with 17-mer PBS primer. Assays were carried out in a 50 uL volume containing 50 mM Tris HCl pH 8.0, 100 ug/mL bovine serum albumin, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 100 nM TP, 100 uM dNTP (25 μM of each of the four dNTPs) with one of them being ^{32} P labeled (1uCi/assay dCTP) and 50 ng/assay enzyme. Reactions were carried out at 37°C for 5 min and were terminated by the addition of ice cold 5% trichloroacetic acid containing 5 mM inorganic pyrophosphate. The samples were filtered on Whatman GF/B filters and processed for radioactivity counting as described before (Lee et al., 1998).

2.3 Gel Analysis of Polymerase Reaction Products

For the gel analysis, 5'-^{32}P labeled 18 mer PBS DNA primer annealed with U5-PBS HIV-1 RNA, 49 mer U5-PBS DNA template and homopolymeric Poly(rA).(dT)18 template primer was used in the polymerase reaction as described above. The primers were end labeled with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P] ATP (3,000 Ci /

mmol) according to the standard protocol (Ausubel et al., 1987). The reaction mixture contained 50 mM Tris HCl pH 8.0, 100 μg/mL bovine serum albumin, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 100 nM TP, 10μM of each of the four dNTPs with one of them being ^{32}P-labeled (0.2 μCi/nmol dNTP) and 50 ng enzyme in a total volume of 5 μL. The reactions were terminated by the addition of 5 μL of Sanger's gel loading dye (Sanger et al., 1977) containing 20 mM EDTA. The extension products were resolved on an 8% denaturing polyacrylamide (7M urea, 1x TBE) sequencing gel.

2.4 ddNTP Sensitivity Assay

The template primers used in this assay included the 49 mer U5 PBS DNA template primed with 32P -labeled 17- mer DNA primer. Samples were incubated at 25°C for 5 min prior to termination using equal volume of Sanger's dye as described before. Final concentrations of dNTP and ddNTP used were 10 μM each. All other conditions were similar to the polymerase assay.

2.5 rNTP Incorporation Assay

The ability of the wild-type and its mutant derivatives to incorporate rNTPs as substrate was assayed on both RNA and DNA templates. The U5 PBS RNA and 49-mer U5 PBS DNA primed with 32-P labeled 17-mer DNA were used as RNA-DNA and DNA-DNA template primer. In each case, the reactions were initiated by the addition of the enzymes in a reaction mixture containing dNTP (20μM final each) or rNTP (100μM final each). The reactions were carried out in a total volume of 5 μL and terminated by the addition of the loading dye as described before. The terminated reaction products were heated for 3

minutes at 90 °C and resolved on an 8% denaturing polyacrylamide gel (7M urea, 1X TBE) sequencing gel.

2.6 Processivity Assay

The processivity of the wild-type RT and its mutant derivatives was assayed using poly (rA) template and 32P-labeled dT18 primer annealed together. The cold poly (rA).(dT)18 was used as a trap at a final concentration of 0.5 μM of primer termini. In reactions where processivity was assayed, enzymes were preincubated with the labeled TP for 30 seconds and then synthesis was initiated by the addition of a solution containing Mg-dNTP and trap. A control reaction was incubated for each enzyme for 5 min of synthesis time, which was not restricted by addition of trap and thus represented maximum synthesis under the given experimental conditions. The efficiency of the trap to bind any enzyme dissociated from the labeled TP was assessed in a reaction where the RNA trap was added before the initiation of the reaction. The molar ratio of the labeled TP to trap was approximately 1: 100 so the probability of an enzyme rebinding to labeled TP was negligible. The final concentration of dNTP was 50μM and the reaction was incubated for 5 minutes at 25°C before terminating it by addition of Sanger's dye and resolving on the denaturing polyacryamide gel.

2.7 Extension of Primers in Presence of Three dNTPs

Labeled 17 mer primer annealed with a three fold excess of U5 PBS RNA was used for determining the extent of misincorporation in the presence of only three dNTPs. The labeled template primer was incubated with 10 ng WT or mutant enzymes at 25°C for 10

min in a total volume of 5μL containing 50 mM Tris -HCL, pH 7.5, 1mM DTT, 0.1 mg of BSA per ml, 5 mM $MgCl_2$ and only three dNTPs at a concenration of 200μM each. (-A= dCTP, dGTP, dTTP; -C = dATP, dGTP, dTTP; -G= dATP, dCTP, dTTP; -T= dATP, dCTP, dGTP) At the end of incubation the reaction was quenched by addition of 5μL of Sanger's gel loading dye. The reaction products were analyzed on a denaturing 10 % polyacrylamide– 8M urea gel.

2.8 Measurement of Pyrophosphorolysis Reaction

Pyrophosphorolysis activity of the mutant enzymes was estimated by analyzing the products of the reaction on denaturing polyacrylamide gels. The U5-PBS HIV-1 RNA and 49-mer U5 PBS DNA templates primed with ^{32}P-labeled 17-mer PBS primer were used for measurement of pyrophosphorolysis reaction. The 6 μL reaction mixture contained 50 μM Tris-HCL, pH 7.5, 1mM DTT, 100 μG BSA, 5mM MgCl2, 1mM sodium pyrophosphate and 10ng enzyme/assay. The reactions were carried out for 60 minutes and quenched by addition of equal volume of Sanger's gel loading dye. The samples were then heated at 95 °C and loaded on denaturing 12% polyacrylamide-8M urea gel. The labeled products were detected by autoradiography.

CHAPTER 3

RESULTS

3.1 Construction and Purification of Mutant Enzymes

Two mutant derivatives of the residue at position 91 of HIV-1 RT were constructed and expressed in *Escherichia coli* by the procedure described before(Pandey et al.,1996; Harris et al., 1998). Two single substitutions were carried out. In the first one, the functional side chain of Gln91 was abolished by replacement with Ala, and in the second one, the replacement was by conservative Asn residue which displays similar geometry and polarity in its side chain as that of Gln. The purified enzyme preparations were found to be homogeneous with a purity of greater than 95%. The level of their expression, solubility and yield as well as the chromatographic characteristics of all the mutant proteins were identical with that of the wild type enzyme suggesting that the substitutions at the mutation sites did not cause any perturbation in the enzyme structure. All the mutants showed a heat inactivation pattern identical to the wild type enzyme (results not shown), thus providing an additional evidence that these mutations had not altered the folded structure of the enzyme protein.

3.2 Polymerase Activity with RNA and DNA Templates

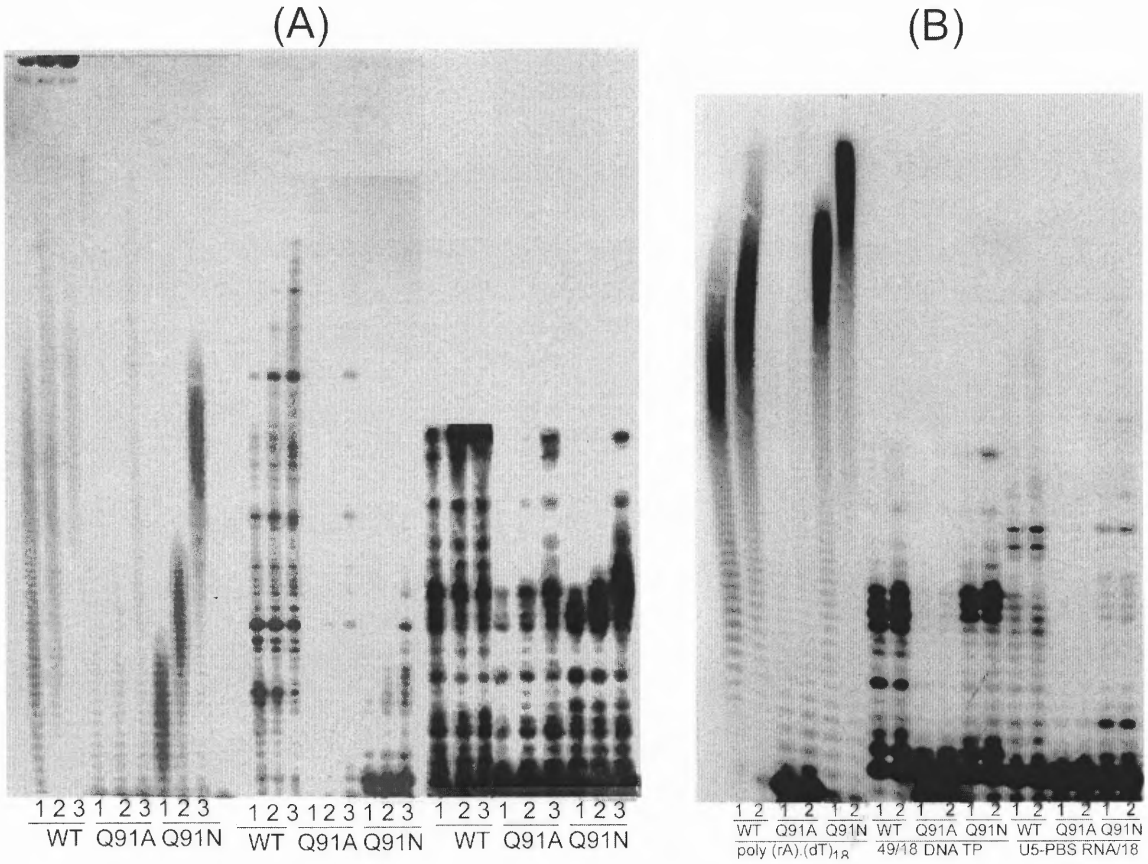

Figure 3.1 RNA and DNA–Directed DNA Polymerase Activity of WT HIV-1 RT and its Mutant Derivatives. Three template -primers poly (rA).$(dT)_{18}$, U5-PBS-RNA/18-mer DNA, and 49-mer U5-PBS DNA/18-mer DNA primer were used to assess the extension reaction catalyzed by the wild-type and mutant derivatives of HIV-1 RT. The primers labeled at the 5' end with ^{32}P were annealed with the respective templates and used in the reactions carried out in a total volume of 6μL as described in Methods. Reactions quenched by the addition of Sanger's dye were analyzed by 10% denaturing gel electrophoresis. Reactions carried out in presence of Mg^{2+} as divalent cation are depicted in panel A and those carried out in presence of Mn^{2+} as divalent cation are depicted in panel B.

A number of mutations of HIV-1 RT have been shown to display differential polymerase activity with different template primers. It has been earlier demonstrated that alanine substitution at position 72 and 151 results in impairment in the polymerase

activity by nearly an order of magnitude with RNA template while only a five-fold reduction was seen with DNA directed reactions (Sarafianos et al., 1995; Kaushik et al., 1997). In order to determine if the mutant derivatives of Gln91 also share the above template effect, we evaluated their polymerase activities with different template primers.

Polymerase activities of the wild-type HIV-1 RT and its mutant derivatives were assessed using poly (rA).$(dT)_{18}$ as well as natural RNA and DNA templates corresponding to the U5 PBS region of HIV-1 genome primed with a PBS primer. The results depicted in Figure 3.1 indicate that both Q91A and Q91N mutant enzymes are severely impaired in their polymerase activity in relation to the wild-type enzyme both RNA and DNA templates. Q91A shows less activity compared with the Q91N mutant enzyme. Interestingly, the extent of primer extension exhibited by Q91N mutant was greatly reduced as compared to the wild type enzyme. Q91N mutant seems to pause more frequently and accumulate shorter products although the degree of initial primer utilization is similar to the wild type enzyme. In contrast, the extension product catalyzed by Q91A is much longer than Q91N mutant despite lower initial utilization of the primer. Notably, the observed 'pausing' pattern of Q91N mutant enzyme is valid on both RNA and DNA templates. The overall pattern exhibited by the Q91N could be compared with the Y183F mutant enzyme albeit with much lower activity (Harris et al., 1998).

Surprisingly, Q91N mutant was capable of exhibiting a significant increase in its polymerase activity when Mg^{2+} was replaced by Mn^{2+} as divalent cation (Figure 3.1B). In fact its polymerase activity seems to be a higher on homopolymeric template than the wild type enzyme in presence of Mn^{2+}. Mutant Q91A, however does not show any substantial improvement in its polymerase activity. These observations hold true with

respect to both homopolymeric or heteropolymeric RNA-DNA and DNA-DNA template-primers. Thus, there seems to be a general improvement in the polymerase activity of Q91N irrespective of the nature of the template used in presence of Mn^{2+}. Improvement of polymerase activity on substitution of Mg^{2+} with Mn^{2+} has been reported in case of other mutant RTs as well. This laboratory has earlier reported similar behavior of mutant derivatives of Asp113, Y183 and Y115 which constitute the dNTP binding pocket of this enzyme (Harris et al., 1998). Similar increase in polymerase activity has been reported by Prasad, et al in their study of E89G mutant enzyme (Prasad et al., 1991).

These results suggest that Mn^{2+} rather than Mg^{2+} might help restore the effect of Q91N mutation by coordinating with the surrounding residues better than for Q91A or Y183F mutant enzyme. Thus, it is possible that the decreased polymerase activity of both Q91A and Q91N may be due to subtle changes in the dNTP binding pocket. Such a change in the pocket may also influence the fidelity, processivity of the enzyme as well as its response to ddNTP inhibitors.

3.3 Sensitivity to ddNTPs

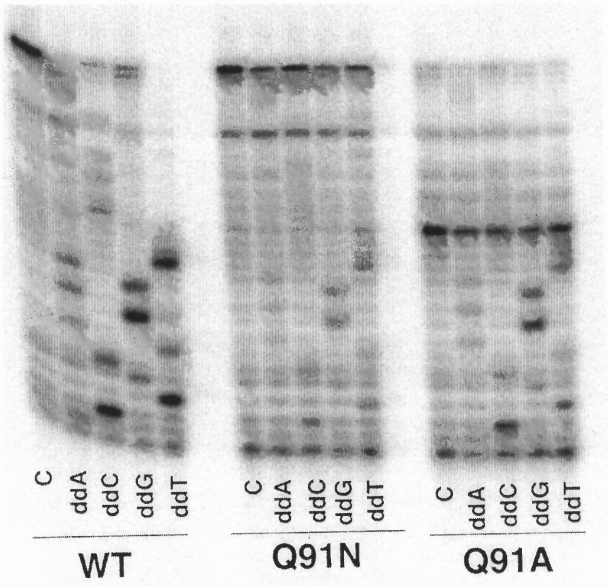

Figure 3.2 Sensitivity of WT HIV-1 RT and its Mutant Enzymes to Dideoxy Nucleoside Triphosphates. Effect of ddNTPs on the DNA synthesis catalyzed by the wild-type and mutant enzymes was assessed on 49-mer U5-PBS DNA template primed with $5'^{32}$-P -labeled 17-mer PBS primer. The final concentration of dNTP and ddNTP was 10μM each. Lane 1 in each set represents extension reaction in the absence of ddNTPs. Lanes 2 to 5 represent extension reaction in presence of ddATP, ddCTP, ddGTP and ddTTP, respectively.

Dideoxynucleotides have been shown to be competitive inhibitors of HIV-1 RT and have also been used for treatment of AIDS. The most potent single agent nucleoside among the approved nucleoside inhibitors is (-)-2'3'-dideoxy-3'thiacytidine (3TC, Lamivudine). However in both cell culture and infected patients, resistance to 3TC develops rapidly. Resistant variants containing an M184I alteration in RT gene appear transiently and then are replaced by those with M184V alteration (Larder, 1995). This M184V mutation has also been shown to confer a low level resistance to ddI and ddC (Drosopoulas et al., 1993). An E89G substitution has been reported to confer multiple resistance to ddNTPs in cell free analysis. In addition this mutation also confers resistance to non-nucleoside RT inhibitors and foscarnet (a Ppi analog) both in-vivo and

in-vitro (Prasad et al., 1991). It has been shown that substitution at 183 position confers resistance to ddNTPs in-vitro (Harris et al., 1998). Since Y183 and Q91 have been proposed to stabilize each other via hydrogen bonding interaction, it was interesting to examine the influence of Q91 substitution on the ddNTP sensitivity of the mutant enzymes as compared to the wild-type enzyme. As depicted in Figure 3.2, both mutations vary in their ability to change response of RT to ddNTP analogs. The sensitivity of Q91A mutant to ddNTP analogs does not significantly differ from the wild type enzyme. In case of Q91N, comparison of lane representing synthesis in absence of ddNTP (lane 1) and in the presence of ddATP, ddCTP, ddGTP and ddTTP (lanes 2-5), respectively, indicates that Q91N substitution confers higher resistance to the ddNTP analogs. These results suggest that the substitution has affected the enzyme activity in a manner similar to that affected by E89G or Y183F alteration.

3.4 Fidelity of the Wild-Type and Mutant Enzymes

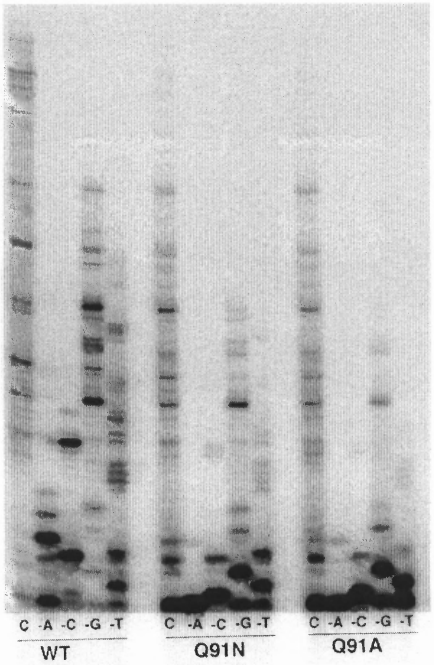

Figure 3.3 Misinsertion and Mispair Extension Catalyzed by the Wild-Type Enzyme and its Mutant Derivative in the Absence of a Single dNTP. Almost equivalent enzymes activities were used in 6μL reactions carried out at 25 degree C for 15 min in presence of all four nucleotides (lane 1), in absence of dATP (lane2), in absence of dCTP (lane 3), in absence of dGTP (lane 4) and in absence of dTTP (lane 5). The extension reactions were analyzed by 10% denaturing polyacrylamide gel electrophoresis as described in the Materials and Methods.

Several previous studies suggest that nucleoside analog resistance could be associated with increased fidelity of HIV-1RT. Mutant enzymes such as M184V (Pandey et al., 1996; Wainberg et al., 1996), E89G (Drosopoulas et al., 1996; Rubinek et al., 1997), M184Leu (Bakhanashvili., et al., 1996) have been shown to exhibit higher enzyme fidelity compared to the wild type enzyme. On the other hand, alterations such as Asn 67, Arg 70 have been shown to exhibit high levels of resistance to nucleoside analogs but decreased enzyme fidelity (Arion et al., 1998; Canard et al., 1998). Hence, ddNTP resistance and fidelity of the enzyme seem to be closely related to and affecting each

other. Thus, it was interesting to examine if Q91A and Q91N mutations influenced these functions of the enzyme. The assay employed here measures the net result of both misinsertion and subsequent mispair extension by omitting a single dNTP from the polymerization reaction.

Results depicted in Figure 3.3 suggest an overall increase in fidelity for both the mutant enzymes compared to the wild type enzyme. Both the mutants are more discriminatory for incorporation of dNTP as well as extension of primers once a misinsertion has taken place as compared to the wild-type.

All these studies suggest a likelihood of both resistance to nucleoside analogs and increase in fidelity might involve similar mechanism. That is the ability to incorporate or reject an incoming dNTP or its analog is accompanied by the ability to recognize the right dNTP for insertion. One might imagine that such high fidelity enzyme might be advantageous for survival of the virus under the selection pressure of drug as it will be less error-prone.

3.5 Incorporation of rNTP versus dNTP

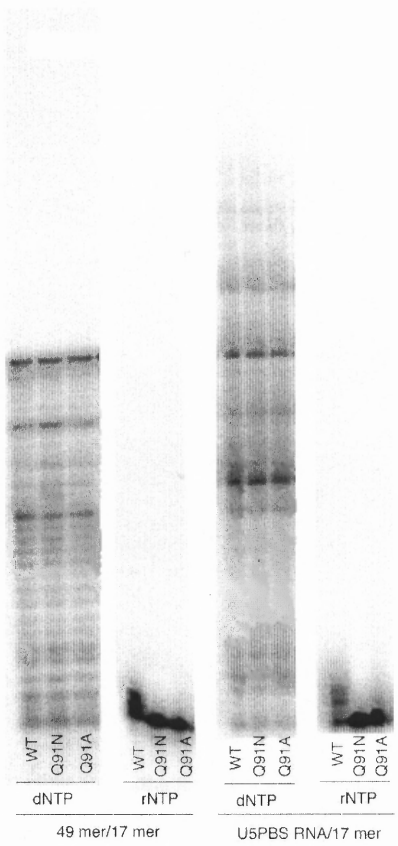

Figure 3.4 Utilization of rNTP in the Extension Reaction Catalyzed by the Wild-Type HIV-1 RT and its Mutant Derivatives. The ability of the RT enzymes to catalyze incorporation of rNTP substrates was examined using U5-PBS-RNA template and 49-mer U5-PBS DNA template each annealed with 5' ^{32}P labeled 17-mer primer. d and r represent extension reactions carried out in presence of 200uM dNTP and 500uM rNTP, respectively. All the reactions were carried out at 25 degree C for 20 min.

High discrimination of mutant enzymes exhibited with respect to incorporation of ddNTP or incorrect dNTP prompted us to study the ability of the enzymes to discriminate between rNTP and dNTP. To assay the ability of mutant enzymes to discriminate between rNTP and dNTP, incorporation of both was studied on primed U5-PBS RNA and U5- PBS DNA templates as described. As is evident from Figure 3.4, none of the mutant RT derivatives incorporate rNTPs as much as the wild-type RT. Both the mutant

derivatives of RT seem to be more discriminatory as compared to the WT enzyme for both DNA and RNA templates.

3.6 Pyrophosphorolysis

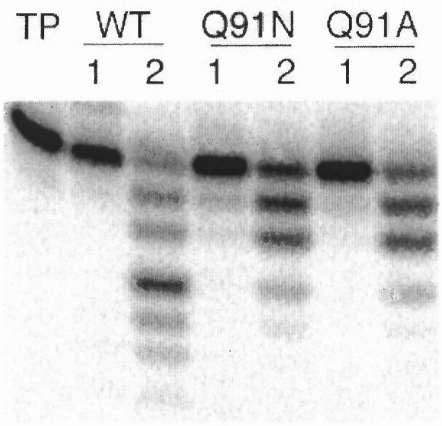

Figure 3.5 Pyrophosphorolysis Reaction Catalyzed by the Wild-Type Enzyme and its Mutant Enzymes. The pyrophosphorolysis activity of the wild-type HIV-1 RT and its mutant derivatives was detected by using heteropolymeric as described in the Materials and Methods. The concentration of pyrophosphate and Mg^{+2} in the reaction mixture was 1mM and 5mM respectively. The reaction products were analyzed on denaturing polyacrylamide-Urea gel as described previously.

Pyrophosphorolysis is the reversal of polymerase activity resulting in cleavage of the DNA primer from the 3' terminus in the presence of PPi and resulting in generation of dNTPs as the products. Pyrophosphorolysis could be very important for survival of HIV -1 under the pressure of drug therapy in vivo in order to remove the chain terminating 3' deoxynucleotides thereby allowing additional chain elongation. Such enhanced pyrophosphorolysis has been reported in HIV1 RT due to mutations D76N/K70R in high level resistance mutant D76N, K70R, T215F, K219Q by Arion, et al. (1998). This laboratory has previously reported that mutations in the residues from dNTP binding

pocket change the pyrophosphorolysis activity of the enzyme in a template dependant manner (Harris et al., 1998). It is possible the observed resistance to ddNTP for Q91A and Q91N mutant enzymes could be due to the increased pyrophosphorolysis activity which can generate 3'-OH by cleaving the ddNTP terminated primer. To examine this possibility, pyrophosphorolysis activity of these mutant enzymes was studied. Both the mutant derivatives displayed a significant amount of pyrophosphorolysis albeit lower than the wild type enzyme (Figure 3.5). This indicates that reduced sensitivity to ddNTP analogs displayed by these mutants is not due to the removal of terminal nucleotide from the chain terminated primer during the synthesis. It has to be noted that both the mutant derivatives are deficient in their polymerase activity. Arion, et al have reported a concomitant increase in the processivity of the mutant enzyme D76N, K70R, T215F, K219Q to compensate for the increased pyrophosphorolysis which is attributed to alterations T215F and K219Q (Arion et al., 1998). Similarly, Canard et al. have proposed that an RT enzyme containing AZT mutation is able to remove selectively an AZT-MP terminated primer-template due to enhanced pyrophosphorolysis (Canard et al., 1998) They further suggested that such a mutant has high affinity for the AZT-MP terminated primer template thus increasing the likelihood of repair at this position. With such a relationship between nucleoside analog resistance, pyrophosphorolysis and processivity it was interesting to study processivity of the mutant derivatives under this study.

3.7 Processivity of WT and its Mutant Derivatives

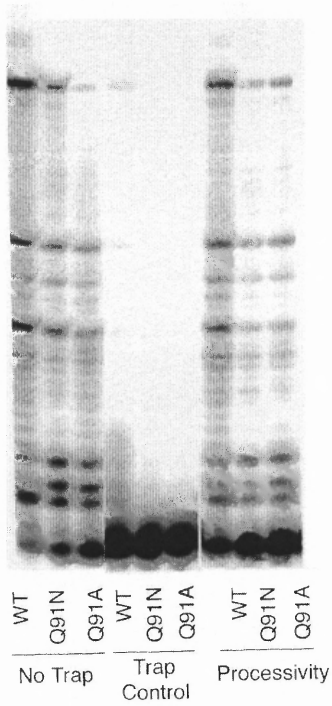

Figure 3.6 Processivity of HIV-1 RT and its Mutant Derivatives U5PBSRNA/17merDNA (TP). (Processivity) - TP was first pre-incubated with RT proteins followed by the addition of trap. The extension reaction was then initiated by adding a mixture containing dNTP and Mg++. (Trap Control) - TP was first mixed with the trap mixture, followed by the addition of RT. The extension reaction was then initiated by the addition of Mg++-dNTP mixture. This condition blocked RT binding to the labeled TP. (No Trap) - TP was premixed with RT proteins and then the extension reaction was initiated by the addition of Mg++- dNTP mixture. This condition allowed multiple rounds of primer extension by RT proteins. All reactions were analyzed by 10% denaturing gel electrophoresis.

Processivity of an enzyme is a measure of the number of nucleotides incorporated into a primer strand during a single enzyme-template-primer binding event before the enzyme dissociates from the template primer. Several mutations of HIV-1 RT have been reported to affect processivity of the enzyme. Drug resistant RT containing the substitution E89G and D76V have been shown to exhibit increased processivity (Kim et al., 1999; Quan et al., 1998). It has been shown that the decreased processivity of Y183F mutant RT could be restored by introduction of a second substitution of Met to Val at

position 184 to the wild-type level (Harris et al., 1998). The processivity of Q91A and Q91N on U5PBS RNA template annealed to 5'^{32}P-labeled 17mer primer was examined as described in the methods. The results as presented in Figure 3.6 indicate that under the assay conditions, Q91N and Q91A appear equally processive. Also, they do not show decrease in processivity compared to the wild-type enzyme.

CHAPTER 4

DISCUSSION

In the 3-D crystal structure of HIV-1 RT, Gln 91 is located on the ß 5a - ß 5b loop of palm subdomain in the polymerase cleft (Kohlstaedt., 1993; Huang et al., 1998). In previous studies from this laboratory, involvement of a residue at position 91 has been suggested through its interaction with the side chain of Y183 - a constituent of highly conserved YXDD motif found in all retroviral reverse transcriptases. Analysis of a 3-D crystal structure based molecular model has indicated that the side chain of Q91 may stabilize the side chain of Y183 through hydrogen bonding interaction (Harris et al., 1998). This prediction was further supported by the mutant modeling in the present studies using the available 3-D crystal structure of HIV-1 RT-DNA-dNTP ternary complex where in one of the energetically favorable conformers, side chains of Y183 and Q91 are positioned within distance appropriate for hydrogen bonding interaction. The abolishment of this interaction by substitution of Tyr to Phe at position 183 resulted in 70% loss of polymerase activity on both RNA and DNA templates with significant increase in the fidelity (Harris et al., 1998). It was expected that loss of hydrogen bonding interaction due to mutation at position 91 may also manifest enzyme characteristic similar to Y183F mutant.

To elucidate the possible role of residue 91 in the HIV-1 RT, two mutations at position 91 were introduced. Mutant enzymes Q91A carrying a non conservative substitution and Q91N with a conservative substitution were studied. As predicted, both substitutions affected the polymerase activity of the enzyme severely. Q91A mutant

enzyme is more impaired in its polymerase activity than the Q91N mutant enzyme. Interestingly, mutant enzyme Q91N shows polymerase activity comparable to the wild type enzyme in the presence of Mn^{2+} as divalent cation. Such results have been previously described for mutations involving residues in the dNTP binding pocket (Harris et al., 1998). These observations suggest that the decreased polymerase activity could be due to the indirect effect of these mutations on the dNTP binding. This study of the sensitivity of the mutant enzymes to the ddNTP analogs shows that both the enzymes are more resistant to all the ddNTPs compared to the wild type enzyme. Enzyme fidelity as studied by the polymerase reaction carried out in absence of one dNTPs suggest higher fidelity of polymerase activity for both Q91N and Q91A mutants in relation to the wild type enzyme. Interestingly, the results obtained for mutant Q91N clearly suggest that its behavior is somewhat similar to both Y183F as well as E89G mutant derivatives of HIV-1 RT(Harris et al., 1998; Prasad et al., 1991). These results also indicated that mutant derivatives at position 91 exhibited marginal reduction in their ability to catalyze pyrophosphorolysis activityof the enzyme. This laboratory has earlier reported differential pyrophoshorolysis activity of RT mutants depending on the nature of the template used (Harris et al., 1998). It was noted that Y183F mutant RT could exhibit equivalent polymerase activities on DNA and RNA templates but was severely impaired in its catalysis of pyrophosphorolysis on DNA template. Interestingly, both the mutants (Q91N and Q91A) were found to be more resistant to ddNTPS than the wild type enzyme. This suggested that any substitution at position 91 may offer greater stringency to the enzyme in the selection of substrate. This may be due to partial loss of non-covalent interaction between the enzyme and the substrate as a result of both mutation

and also modification of the substrate. This may result in reduced flexibility of the dNTP binding pocket which may decrease the overall binding energy released from these weak interactions. Although release of optimum binding energy is essential for lowering the activation energy of the reaction, it also offers greater flexibility to the substrate binding pocket. The increased resistance to ddNTP exhibited by both the mutants may be due to reduced flexibility of their dNTP binding pocket. This contention was also supported by the fact that both the mutant derivatives displayed higher fidelity as compared to the wild type enzyme. However, between the two mutants, Q91N mutant derivatives of HIV 1 RT is characterized by increased resistance to ddNTP analogs, and preferred usage of Mn^{2+} instead of Mg^{2+} as divalent cation for polymerase activity.

These characters are similar to the E89G mutant enzyme derivative that has been isolated from HIV-1 infected cell culture (Prasad., et al., 1991). However, E89G mutant enzyme has been shown to posses higher processivity that probably compensates the decreased forward synthesis due to decreased polymerase activity and increased fidelity (Kim et al., 1999). An enzyme with increased pyrophosphorolysis compared to the polymerase activity and decreased processivity could be detrimental to viral replication *in vivo*. Increased fidelity tends to slow down the forward reaction further. We expect that such a mutation may have difficulty surviving *in vivo*. Indeed, mutation at this position has not so far been reported in HIV-1 variants isolated from AIDS patients or from HIV-1 infected cell cultures. This supposition has been well supported by studies of Tachedjian, *et al* (Tachedjian et al., 1996). While studying an inverse nature of interaction between AZT resistant and PFA resistant mutants *in vivo*, they intended to introduce PFA resistance notably, one involving residue 89 (E89K) into engineered AZT

resistant background. Their attempts to recover infectious viruses containing mutation Q91L were not successful and they concluded that the said mutation might not be viable. Interestingly, the same authors could readily isolate E89K as a foscarnet (phosphonoformic acid, PFA), resistant mutation from MT-2 cells exposed to increasing concentrations of the drug. This drug is a PPi analog used to treat infections due to cytomegalo virus (such as retinitis) in AIDS patients. The Antiretroviral effect of PFA was one of the suggested explanations for improved survival of PFA treated patients in a study that compared PFA with ganciclovir for treatment of CMV retinitis in AIDS patients (Crumpacker, 1996; Oberg, 1989). Mutation E89G has been reported to be resistant to PFA along with ddTTP, ddCTP, ddATP, ddGTP, AZTTP and 3TCTP. This resistance and its increased processivity have been attributed to the interaction of Glu 89 with the penultimate nucleotide of the double stranded template (Drosopoulos et al., 1996).

All these results point towards a possible involvement of residue 89 and 91 in the pyrophosphorolysis activity of the HIV-1 RT probably via their interaction with the template primer duplex. For any catalytically important residue for an enzyme activity, it is expected to be fairly well conserved in sequences carrying out similar functions in nature. Xiong and Eichenbush have studied the origin and evolution of retro-elements based on their reverse transcriptase sequences (Xiong., et al 1990). They have noted that Q at position 91 has been conserved in 17 out of 29 retroviral sequences which is more than 60 %. The same is very well conserved in the related viruses HIV-1, HIV-2 and SIV (Korber et al., 1998). In the rest of the retroviral sequences reported, it is either serine (5 out of 29) or valine (6 out of 29). In case of Human Spuma retrovirus, it is asparagine.

Interestingly, although the neighboring residues of 91 have been implicated in drug resistance, there have been no reports on involvement of 91 in any functional aspects of HIV 1 RT. Mutations such as Trp 88 Gly/Ser (Mellors et al., 1995), Glu89 Gly/Lys (Prasad et al., 1991), Leu 92 Ile (Tachedjian et al., 1995) have all been implicated in the resistance to foscarnet (PFA)- a pyrophosphate analogue used to treat AIDS patients. Although these are located rather away from the putative pyrophosphate binding site, their effect is probably exerted by an altered interaction of the mutant enzyme with the template strand distorting the geometry of the polymerase active site and thereby decreasing PPi binding. Absence of reports on involvement of residue 91 in drug resistance might indirectly imply mutation at this position may not be viable. This postulation is supported from the studies of Tachedjian, *et al* (Tachedjian et al., 1996) where HIV-1 carrying Q91L mutation in the RT gene was found to be noninfectious and replication incompetent. Additionally, results from our own laboratory suggest that mutations at highly conserved neighboring positions 94 and 95 are extremely deleterious to the polymerase activity of the HIV 1 RT.

In a 3-D crystal structure of the RT-DNA-dNTP ternary complex residue 89 is positioned near the phosphate back bone of the second template nucleotide in the duplex region, whereas residue at position 91 is within interacting distance from the side chain of Gln 161 and sugar moiety of 3rd template nucleotide in the duplex region. In order to analyze the inter-atomic interactions, we carried out mutant modeling of Q91N in the 3D crystal structure of the ternary complex (Huang et al., 1998). We observed significant variations in the side chain conformation of E89, in the mutant enzymes as compared to

in the wild type enzyme. In the wild type enzyme, the side chain of K154 is in a position to make salt bridge interactions with both phosphate group of template nucleotide and the side chain of E89 while in the mutant enzymes (Q91N, Q91A), E89 is farther from K154 but closer to the phosphate group of 2nd template nucleotide. This putative interaction presents a very interesting scenario during the polymerase reaction. It is possible that in the wild type enzyme, E89 is transiently interacting with K154 to destabilize its salt bridge interaction with the phosphate group of template nucleotide and thus facilitating the translocation of enzyme following each cycle of polymerization. In the mutant enzymes, the salt bridge interaction between K154 and template nucleotide seems to be more stable due to lack of interaction between E89 and K154 may cause enzyme remaining in locked-in position resulting in strong pausing and subsequent translocation following each polymerizing event. The reduction in the length of the side chain at position 91 also results in the loss of hydrogen binding interaction with the side chain of Y183. A significant change in the orientation of Q161 side chain was also noted. Q161 of βE is located on conserved region very near to the dNTP and primer terminus but is not solvent accessible in the RT-DNA binary complex (Jacobo Molina et al., 1993). However, in the ternary complex, the side chain of Q161 may undergo conformational change to influence the geometry of dNTP binding pocket. A conformational search of Q91 and N91 side chain in the wild type and mutant model has shown a very interesting pattern of interaction with Q161 and template strand. In case of wild type enzyme (that is with Q at position 91) out of 21 allowed conformations, only six were within the interacting distance from both Q161 (2.7 Å –3.1Å) and 3rd template nucleotide (2.8 Å-3.1 Å). In contrast, the conformational search of N91 yielded 9 allowed conformations

all of which were within interacting distance from Q161 (2.7 Å – 3.5 Å) but farther from the template strand. These observations suggest that stabilization of duplex region of template-primer may be affected by Q to N substitution at position 91. The present study as well as our previous observations (Harris et al., 1998) seems to suggest that Q91 and Q161 together with Y183 are also important constituents of the dNTP binding pocket as judged by greater discriminatory property of Q91N and Y183F mutants against rNTP and dNTP substrates.

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