Published in final edited form as:

Antiviral Res. 2015 March ; 115: 75–82. doi:10.1016/j.antiviral.2014.12.016.

Pre-steady State Kinetic Analysis of HIV-1 Reverse Transcriptase for Non-canonical Ribonucleoside Triphosphate Incorporation and DNA Synthesis from Ribonucleoside-containing DNA Template

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Abstract

Non-dividing macrophages maintain extremely low cellular deoxyribonucleotide triphosphate (dNTP) levels, but high ribonucleotide triphosphate (rNTP) concentrations. The disparate nucleotide pools kinetically forces Human Immunodeficiency Virus 1 (HIV-1) reverse transcriptase (RT) to incorporate non-canonical rNTPs during reverse transcription. HIV-1 RT pauses near ribonucleoside monophosphates (rNMPs) embedded in the template DNA, which has previously been shown to enhance mismatch extension. Here, pre-steady state kinetic analysis shows rNTP binding affinity (K_d) of HIV-1 RT for non-canonical rNTPs was 1.4- to 43-fold lower, and the rNTP rate of incorporation (k_{pol}) was 15- to 1551-fold slower than for dNTPs. This suggests that RT is more selective for incorporation of dNTPs rather than rNTPs. HIV-1 RT selectivity for dNTP versus rNTP is the lowest for ATP, implying that HIV-1 RT preferentially incorporates ATP when dATP concentration is limited. We observed that incorporation of a dNTP occurring one nucleotide before an embedded rNMP in the template had a 29-fold greater K_d and a 20-fold slower k_{pol} as compared to the same template containing dNMP. This reduced the overall dNTP incorporation efficiency of HIV-1 RT by 581-fold. Finally, the RT mutant Y115F displayed lower discrimination against rNTPs due to its increase in binding affinity for non-canonical rNTPs. Overall, these kinetic results demonstrate that HIV-1 RT utilizes both substrate binding

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and a conformational change during: 1) enzymatic discrimination of non-canonical rNTPs from dNTPs and 2) during dNTP primer extension with DNA templates containing embedded rNMP.

Keywords

HIV-1; Reverse transcriptase; kinetics; dNTPs; rNTPs; Macrophages

1. INTRODUCTION

When HIV-1 infects a cell, the viral DNA polymerase HIV-1 reverse transcriptase (RT) enzymatically converts viral, single-stranded, genomic RNAs into double-stranded, proviral DNA using its RNA- and DNA-dependent DNA polymerase activities. HIV-1 and other lentiviruses have the unique ability to replicate in both actively dividing CD4⁺ T cells and non-dividing, terminally differentiated macrophages. We previously reported that non-dividing macrophages contain extremely low cellular dNTP concentration (20–40 nM), compared to activated CD4⁺ T cells (1–16 μ M) (1,2). This paucity of dNTPs suppresses HIV-1 reverse transcription kinetics in macrophages (1,3,4). Nonetheless, HIV-1 RT displays a very low K_m value that enables the virus to complete proviral DNA synthesis even in non-dividing macrophages (1,2). However, the K_m value of HIV-1 RT is still above the dNTP concentration found in macrophages, which explains the slower viral replication kinetics in macrophages as compared to activated CD4⁺ T cells. Recent studies revealed that the host sterile alpha motif and HD domain 1 (SAMHD1) protein, which hydrolyzes cellular dNTPs, is responsible for the extremely low dNTP concentration observed in macrophages (4–6). Indeed, SAMHD1 restricted the HIV-1 reverse transcription step in macrophages through dNTP hydrolysis activity, which also leads to higher viral recombination events in macrophages (3,4,7). Interestingly, other lentiviruses, such as Human Immunodeficiency Virus 2 (HIV-2) and some Simian Immunodeficiency Virus (SIVs), encode a viral accessory protein called viral protein X (Vpx), which counteracts the antiviral SAMHD1 function by targeting SAMHD1 for proteosomal degradation (6,8,9). The degradation of SAMHD1 leads to the elevation of cellular dNTP concentrations and rescues the delayed viral DNA synthesis kinetics in macrophages (3,4).

However, unlike dNTPs, cellular rNTP levels are maintained in the millimolar ranges in both activated CD4⁺ T cells and macrophages (2). Both cell types require abundant rNTPs for RNA synthesis and cell signaling kinases, in addition to acting as an energy carrier (10,11). Interestingly, the discrepancy between dNTP and rNTP concentrations in macrophages is higher than compared to activated CD4⁺ T cells because of the extremely low dNTP concentrations in macrophages (2). This large disparity between rNTP and dNTP substrates in macrophages kinetically forces HIV-1 RT to incorporate non-canonical rNTPs during proviral DNA synthesis (2,12). Indeed, we recently demonstrated that HIV-1 incorporated non-canonical rNTPs during reverse transcription in monocyte derived macrophages (2,13). However, rNTP incorporation into HIV-1 proviral DNA was not observed in activated CD4⁺ T cells, likely because of high dNTPs levels (2,13). Our previous experiments demonstrated that HIV-1 incorporated non-canonical rNTPs during proviral DNA synthesis in non-dividing macrophages at a rate of 1/146 nucleotide, implying

that a total of approximately 140 rNTPs can be incorporated into HIV-1 viral DNA during each infection cycle in non-dividing macrophages (13).

rNTP incorporation during DNA replication in yeast has been shown to be mutagenic and to cause genome instability (14–16). An embedded rNMP in a DNA template increases the pausing of cellular DNA polymerases, which is associated with increased mutation during DNA synthesis (17,18). Organisms commonly harbor repair mechanisms that specifically remove rNMPs incorporated in dsDNA, most likely to avoid genomic mutations (13,16,19,20). We recently reported that HIV-1 RT also paused near rNMP sites in the DNA template during DNA synthesis (17).

Most DNA polymerases, including DNA polymerases β , γ , and ϵ , have highly conserved steric gate residues that discriminate rNTPs from dNTPs in order to prevent rNTP incorporation during DNA synthesis (14,15,19,21–23). The ability of HIV-1 RT to restrict rNTP incorporation is based on a bulky tyrosine at residue 115 within the dNTP binding pocket, which sterically clashes with the 2'-OH group of an incoming rNTP (24,25). Mutating this residue to valine or glycine, which possess smaller side chains, increases rNTP incorporation (24,25). An amino acid substitution at this position (Y115F) for HIV-1 RT has also been associated with low-level resistance to several nucleoside reverse transcriptase inhibitors (NRTI) (26–28). Steady state kinetic studies report that wild type HIV-1 RT has a K_m value of more than 100-fold higher for rNTPs than for dNTPs (1,2). The k_{cat} , which measures the turnover number of HIV-1 RT for rNTPs was 10-fold lower when compared to dNTPs (2). However, the pre-steady state kinetic analysis for HIV-1 RT rNTPs incorporation has yet to be determined. Moreover, it is unknown how an rNMP embedded in a DNA template induces the HIV-1 RT pausing. Substituting phenylalanine for the RT steric gate residue Y115 (i.e. Y115F) decreases RT's ability to discriminate rNTPs from dNTPs, which warrants further pre-steady state kinetic investigation. In this report we employed pre-steady state kinetic analysis to measure substrate binding affinity (K_d) and substrate incorporation rate (k_{pol}) to mechanistically explain usage of non-canonical rNTPs by HIV-1 RT during proviral DNA synthesis in non-dividing macrophages.

2. MATERIALS AND METHODS

2.1 RT purification

Heterodimeric RT was cloned from HXB2 HIV-1 strain with the p66 subunit cloned into a pET28a expression plasmid from Novagen. The p51 subunit was cloned into a pCDF expression plasmid with an N-terminal hexa-histidine tag. The Y115F mutation was introduced into the p66 subunit using a QuikChange Site-Directed Mutagenesis Kit from Agilent Technologies. Both p66/p51 subunits were co-expressed in *E. coli* BL-21 (DE3) and purified on a Ni-NTA chromatography column from Novagen. Heterodimer purification was performed as previously described (2,29).

2.2 Nucleotide triphosphates and other materials

A dNTP set and NTP set were obtained from Thermo Fisher Scientific. 3'-deoxycytidine-5'-triphosphate (3'dCTP) was obtained from TriLink BioTechnologies. [γ - 32 P] ATP was obtained from PerkinElmer.

2.3 Pre-steady state kinetic analysis of HIV-1 RT protein

Single nucleotide incorporation burst experiments were used to determine the percent of active RT proteins after purification. DNA primer named “Extend T” (5'-CCGAATTCCCGCTAGCAATATTC) was labeled using [γ - 32 P] ATP and annealed to a 38-mer RNA template (5'-GCUUGGCUGCAGAAUAUUGCUAGCGGGAAUUCGGCGCG) from Dharmacon Research at 4 μ M: 8 μ M. An unlabeled “Extend T” DNA primer was annealed to a 38-mer RNA template described above at 4 μ M: 8 μ M respectively to make the cold pre-annealed primers. The reaction was initiated using HIV-1 RT pre-incubated with template/primer (T/P) and the addition of 800 μ M of dTTP substrate with 10 mM MgCl_2 . For Y115F RT, a 24-mer DNA primer (5'-TCAGGTCCCTGTTCGGGCGCCACT) was annealed to a 36-mer DNA template (5'-TCTCTAGCAGTGGCGCCCGAACAGGGACCTGAAAGC), and this P/T pair was used to measure dGTP single nucleotide incorporation. HIV-1 RT 100 nM final concentration was used in each reaction to extend 100 nM of hot and 200 nM of cold pre-annealed primers. HIV-1 WT RT was preincubated with reaction mixture containing T/P for 3 minutes. Substrate was added to the Kintek Instruments Model RQF-3 rapid-quench flow apparatus and mixed with 10 mM MgCl_2 , and 300 μ M dNTP before stopping the reaction with 0.5 M EDTA at various time points from 0.005 to 3 seconds. Extended products were resolved on a 16% denaturing gel and read with a phosphorimager from Biorad. Quantity One 1-D analysis software from Biorad was used to determine the percent extension from each reaction. Data were fitted to a burst equation 1: $[\text{Product}] = A[1 - \exp(-k_{\text{obs}}t)] + (k_{\text{ss}}t)$ where A is the amplitude of the burst, k_{obs} is the observed first-order rate constant, and k_{ss} is the linear steady-state rate constant (30–32).

2.4 Pre-steady state kinetic analysis of HIV-1 RT rNTP and dNTP incorporation

Single turnover assays of each rNTP and dNTP were performed to assess the K_d and k_{pol} values. Specific 23-mer primers were used to incorporate their corresponding base and are listed as follow: “Extend A” (5'-CGCGCCGAATTCCCGCTAGCAAT), “Extend T” (5'-CCGAATTCCCGCTAGCAATATTC), “Extend C” (5'-GCCGAATTCCCGCTAGCAATATT), “Extend G” (5'-CGAATTCCCGCTAGCAATATTCT). These primers were [γ - 32 P]ATP labeled at the 5' end and annealed to a 38-mer RNA template with the ratio described above. 3'dCTP pre-steady state analysis was completed utilizing extend C primers and the template above. To assess the pre-steady state kinetics of dNTP single nucleotide incorporation before, across and after an embedded rNMP, a 21-mer 3'ACGCGCCGAATTCCCGCTAGCA, 22-mer 3'ACGCGCCGAATTCCCGCTAGCAA, and 23-mer 3'TCGCGCCGAATTCCCGCTAGCAAT were [γ - 32 P]ATP labeled and annealed to 48-mer 23 dA template CGA GCT AAG CGC TTG ACC GCA GAA CAT TGC TAG CGG GAA TTC GGC GCG or 48-mer 23 rA template CGA GCT AAG CGC TTG ACC GCA GAA CAT TGC TAG CGG GAA TTC GGC GCG (17). The Kintek apparatus ran the reactions as previously described (32). The data were fitted to a non-linear regression curve equation 2: $k_{\text{obs}} = k_{\text{pol}}[\text{dNTP}] / (K_d + [\text{dNTP}])$ where k_{obs} is the observed pre-steady state burst rate, k_{pol} is the maximum rate of incorporation, and K_d is the equilibrium dissociation constant for the dNTP (30,31,33).

3. RESULTS

3.1 Pre-steady state kinetic analysis on HIV-1 RT rNTP incorporation

We employed a pre-steady state kinetic analysis using a rapid quench instrument to determine the binding affinity (K_d) and rate of incorporation (k_{pol}) of rNTPs by HIV-1 RT during a single round of primer extension. First, the active site concentration of purified wild type HXB2 HIV-1 RT protein was determined using a ^{32}P -labeled 23-mer T primer annealed to the 38-mer RNA template. Determination of the active site concentration of the RT proteins ensured that 100 nM of active enzyme was put into each reaction for a single turnover of substrate incorporation from prebound RT protein on T/P. The active site concentration determination is shown in Fig. 1A. HIV-1 RT (100 nM) was prebound to 300 nM T/P, and product formation was assessed by adding 800 μM dTTP from 5 ms to 3 s. Typically, a burst of extended T/P from the prebound RT (burst phase) is formed by one round of substrate incorporation. Then it is followed by a more gradual formation of extended T/P (steady state phase), which occurs when RT can conduct multiple rounds of substrate incorporation due to the excess T/P. The results were fitted to the burst equation 1 from Materials and Methods 2.3, and the amount of active RT was determined. We observed on average 80% of the purified wild type HXB2 heterodimer RT protein was active (Fig. 1A).

Next, rNTP incorporation by HIV-1 RT was studied using single turnover experiments in which excess RT is incubated with T/P to start the reaction. This was performed for all four rNTPs to determine the K_d and k_{pol} values. As displayed in representative curves in Fig. 1B and Fig. 1C, ATP (circles) and dGTP (triangles), respectively, showed a much faster rate of incorporation (k_{pol}) as compared to dCTP (diamonds) and dTTP (squares). These data were fitted using equation 2 in Materials and Methods 2.4 to generate the K_d and k_{pol} values of HIV-1 RT for the four rNTPs (Table 1), as well as our measured values of HIV-1 RT with four canonical dNTPs under the same experimental conditions. WT RT pre-steady state kinetics values for ribonucleotide incorporation of ATP, GTP, CTP, and UTP are displayed in Table 1. The k_{pol} values for rNTP incorporation were 15- to 1551-fold slower than dNTP incorporation as shown in Table 1. The k_{pol} values from fastest to slowest incorporation rates were as follows: ATP > GTP > CTP > UTP. For K_d values, HIV-1 RT has 1.4- to 43-fold weaker binding affinity for rNTPs than for dNTPs. The HIV-1 RT K_d values from the lowest (highest binding affinity) to highest (lowest binding affinity) are: ATP > GTP > UTP > CTP. ATP had the overall highest nucleotide incorporation efficiency (k_{pol}/K_d) for HIV-1 RT, and CTP the lowest. Our pre-steady state kinetic results explain the previous steady state analysis from Kennedy *et al.* 2010, which also showed RT to have the highest incorporation efficiency for ATP (2).

We next calculated the selectivity of HIV-1 RT for dNTP versus rNTP by dividing the dNTP incorporation efficiency by the rNTP incorporation efficiency to determine the likelihood that RT would incorporate a dNTP rather than an rNTP. As shown in Table 1, HIV-1 RT displayed the highest selectivity against UTP and lowest against ATP. These results suggest that HIV-1 RT efficiently differentiates all four non-canonical rNTPs from their corresponding dNTPs in regards to both K_d and k_{pol} . Furthermore, not only is the

cellular ATP concentration in human primary macrophages the highest among the four rNTPs, but ATP (1.1 mM) and dATP (40 nM) have the largest concentration difference at more than 28,000-fold (2). The enzymatic preference for ATP incorporation, and the large disparity between dATP and ATP concentrations in macrophages would strongly force HIV-1 RT to incorporate ATP during reverse transcription in macrophages.

3.2 The kinetics of dNTP incorporation with a template containing an embedded ribonucleoside

Incorporation of non-canonical rNTPs by HIV-1 RT during (–) strand DNA synthesis generates DNA templates containing ribonucleoside monophosphates (rNMPs), which are known to induce pausing of both host DNA polymerases and viral HIV-1 RT (17,20). HIV-1 RT can pause during the (+) strand proviral DNA synthesis due to the rNMP-containing (–) strand proviral DNA template (17). Interestingly, during processive steady state DNA synthesis by HIV-1 RT with an rNMP-containing DNA template, the pause product was observed at two nucleotides before the rNMP sites, implying that the dNTP incorporation efficiency was diminished at one nucleotide before the rNMP site (position -1) (16). Thus, three different primers were generated and tested. We determined the pre-steady state kinetic values of dNTP incorporation at three different nucleotide sites within the DNA template: 1) one nucleotide before the rNMP site (position -1), 2) after the rNMP site (position +1), and 3) at the exact rNMP site (position 0) (Fig. 2). The 38-mer DNA template named “dA template” with the identical nucleotide sequence but lacking the rNMP was used as a control template (17). As shown in Fig. 2, the nucleotide sites incorporated by HIV-1 RT are indicated with respect to the position of the embedded rAMP (rA Template). The 21-mer primer represents incorporation of dATP opposite dTMP (dT), which is at one nucleotide before the embedded rAMP in the 38-mer DNA template (position -1). The 22-mer represents dTTP incorporation across from the embedded rAMP (rA) in the DNA (position 0), and the 23-mer indicates dGTP incorporation paired to dCMP (dC) (position +1) after the rAMP site.

Table 2 shows the pre-steady state kinetic values of dNTP incorporation with the rA template and the control dA template. The 21-mer primer extension (position -1) with dATP using the rA template shows a K_d of $50.74 \pm 4.77 \mu\text{M}$ and a k_{pol} of $1.86 \pm 0.08 \text{ s}^{-1}$, which leads to a 20-fold slower rate of incorporation and binds to dATP with a 29-fold lower affinity than the same extension reactions with the control dA template. However, as shown in Table 2, incorporation of dTTP at the position 0 of the rA template shows a K_d that is 10-fold higher and a 4-fold higher k_{pol} value than compared to dTTP incorporation across from the control dA template. This results in a minimal (2-fold) reduction of the dTTP incorporation kinetics across from an embedded rAMP as compared to normal dAMP. This is consistent with no significant pauses seen for HIV-1 RT at the site directly opposite to the rNMP site (position 0) during processive steady state DNA synthesis (17). Finally, the dGTP incorporation using the 23-mer at position +1 with the rAMP-containing template shows a 3-fold lower K_d value and a similar k_{pol} value as compared to the control dAMP containing template. Taken together, we found that while the rAMP embedded in the DNA template induces only minimal kinetic alterations at positions 0 and +1, this embedded non-canonical rAMP significantly deters DNA synthesis kinetics by decreasing the incorporation

efficiency at position -1 by 581-fold, which results from the reduction of both dNTP binding affinity and incorporation rate.

3.3 Comparison of the pre-steady state kinetic parameters of wild type and Y115F HIV-1 RT proteins

Most DNA polymerases have a conserved mechanism to prevent the incorporation of the highly abundant rNTPs during DNA synthesis. This discrimination involves a “steric gate” amino acid residue with an aromatic bulky side chain at the active site of the polymerase, usually tyrosine (19,21,23,34). HIV-1 RT also encodes this tyrosine at position 115 as a steric gate residue. Mutations in Y115 to a smaller side chain (e.g. Y115A, Y115V) reduce RT’s ability to gate the active site and to exclude rNTP incorporation (24,25,35). HIV-1 RT contains the highly conserved Y115 residue, and mutations in this residue are reported to increase rNTP incorporation(24,25,35). However, the mechanism of how mutations in Y115 increase rNTP incorporation remains elusive.

With this in mind, we determined the pre-steady state K_d and k_{pol} values of Y115F HIV-1 RT for dCTP and CTP (Fig. 3A) and compared them with those of wild type HIV-1 RT. We purified Y115F RT with 40% active concentrations which displayed a biphasic burst of product formation suggesting that the mechanistic pathway was not changed by the mutant RT (Data not shown). From Table 3, the pre-steady state kinetic values of Y115F RT display little differences in K_d and k_{pol} values during the canonical dCTP incorporation, resulting in nearly equal overall dCTP incorporation efficiency when compared with WT RT. However, when the K_d and the k_{pol} values were measured with non-canonical CTP, the Y115F mutant showed an equal k_{pol} value and a 13-fold lower K_d as compared to wild type RT. Y115F RT’s selectivity for dCTP over CTP was 1714 ± 297.4 , which suggests that WT RT has a 10-fold higher selectivity for dCTP over CTP when compared with Y115F RT. Selectivity between Y115F and WT RT for CTP was the highest, and not surprisingly, the lowest selectivity was for dCTP.

These data demonstrate that the Y115F RT efficiently incorporates non-canonical CTP by exclusively increasing its binding affinity to CTP. Collectively, our data support that an alteration in the residue Y115 to Y115F allows non-canonical CTP easier access to the substrate-binding pocket of Y115F HIV-1 RT. We suspect that the hydroxyl group in Y115 may clash with 2'-OH group of an incoming CTP leading to the 13-fold difference we see in binding affinity for WT versus Y115F RT.

3.4 3'dCTP chain terminator incorporation by wild type and Y115F HIV-1 RT proteins

Previously, it was shown that ribonucleoside chain terminators (rNCTs), which contain a 2'-OH, but lack a 3'-OH (i.e. 3'dCTP, Fig. 3A) could inhibit HIV-1 replication specifically in macrophages (1). HIV-1 RT incorporates cellular rNTPs only at low dNTP (the canonical substrate) concentrations, which occurs in non-dividing cells like macrophages (2). Since our data in Table 1 demonstrated that the 2'-OH of rNTPs negatively affected both K_d and k_{pol} parameters during rNTP incorporation by RT, we decided to test whether the lack of a 3'-OH on rNTPs also affects the kinetics of incorporation. As such, we determined the pre-steady state kinetic values of wild type HIV-1 and Y115F RT enzymes with 3'dCTP (Fig.

3B), and compared them with the values for dCTP and CTP. As shown in Table 3, WT RT shows an increase in its K_d value and a decrease in k_{pol} value with 3'dCTP as compared to canonical dCTP. However, when we compared the values of CTP and 3'dCTP (Table 3), 3'dCTP binds 15-fold more tightly to WT RT than CTP even though both substrates contain 2'-OH. This suggests that the absence of a 3'-OH may enable a 2'-OH to avoid the steric clash with Y115 residue. However, WT RT with a 3'dCTP substrate had a lower k_{pol} value than CTP substrate suggesting that the presence of a 2'-OH and the loss of a 3'-OH group in the substrate lowers the incorporation rate. The Y115F RT enzyme displayed a 12-fold higher catalytic efficiency for 3'dCTP incorporation than WT RT. The higher catalytic efficiency of Y115F RT for 3'dCTP incorporation is due to a 6-fold tighter binding affinity to the substrate compared to wild type RT. The 3'dCTP selectivity ratio (k_{pol}/K_d ratios for dCTP vs. 3'dCTP) of these two proteins demonstrates that the Y115F RT enzyme discriminates against 3'dCTP 12-fold less effectively than wild type RT (Fig. 3C), likely due to the loss of the hydroxyl group on tyrosine.

4. DISCUSSION

HIV-1 replication kinetics heavily rely on the availability of the cellular dNTP substrate for proviral DNA synthesis. The concentrations of dNTPs are vastly different between the two major viral target cell types, non-dividing macrophages and activated CD4⁺ T cells. Due to the lack of cell cycling and synthesis of chromosomal DNA, terminally-differentiated, non-dividing macrophages harbor extremely low dNTP levels as compared to activated CD4⁺ T cells (1,2). However, unlike cellular dNTP levels, rNTP levels are equally high in both cell types, up to the millimolar range, presumably because rNTPs are substrates for various cellular enzymatic reactions such as transcription, kinase signaling and energy metabolism (10,11). Indeed, the extremely low dNTP concentrations in macrophages make the disparity between rNTP and dNTP concentrations much greater when compared to activated CD4⁺ T-cells (2).

HIV-1 maintains the ability to replicate in low cellular dNTP concentrations due to the high binding affinity of HIV-1 RT to dNTPs (1,2,36). However, the extremely low level of canonical dNTPs and high abundance of the non-canonical rNTPs kinetically forces HIV-1 RT to incorporate rNTPs during proviral DNA synthesis in macrophages. As shown in Table 1, HIV-1 RT displays higher K_d and lower k_{pol} values for the non-canonical rNTPs as compared to dNTPs. This indicates that not only the access to the binding pocket is severely restricted for rNTPs, but also the conformational change/incorporation step, which occurs after rNTP binds, is also restricted as compared to dNTPs. Possibly the rNTP sitting in the binding pocket of HIV-1 RT may not position properly, which lowers the conformational change rate and/or chemical reaction for the phosphodiester bond formation. HIV-1 RT has the lowest selectivity between dATP and ATP among the four nucleotide-pairs. More interestingly, we previously reported that the cellular ATP concentration is the highest among four rNTPs in macrophages, and the disparity between ATP and dATP concentrations in macrophages is the largest among the four nucleotide-pairs (2). These observations strongly suggest that ATP is the predominant, incorporated rNTP during HIV-1 proviral DNA synthesis in non-dividing macrophages.

Our recent steady state kinetic study showed that an rNMP embedded in the DNA template can cause RT pausing during reverse transcription, and pausing has been associated with decreased HIV-1 RT fidelity near the pause site. This same phenomenon is also observed for other DNA polymerases (17,18). Data in Table 2 show that RT is 581-fold less efficient in incorporating dATP at the position -1 from the embedded rAMP in the template compared to the control dAMP containing template. Our data demonstrate that the embedded rAMP induces both a slow rate of incorporation and a weak binding affinity to dNTP substrate at the position -1, which mechanistically explains the HIV-1 RT pausing at position -1 during steady state processive DNA synthesis (17). Structural studies demonstrate that the rNMP containing double-stranded DNA displays an altered local structure near the rNMP site, and this template structural change appears to influence both K_d and k_{pol} steps of HIV-1 RT at position -1 (17,37,38). Nonetheless, the rNMP induced structural change in the template does not affect the RT kinetics at position 0 implying that the structural impact is very local and site specific.

As shown in Table 3, the Y115F RT enzyme incorporates CTP more efficiently than WT RT by specifically increasing the binding affinity to CTP by 13-fold without compromising either the binding to the canonical dCTP substrate or the conformational change/chemistry step. However, unexpectedly, 3'dCTP binds to WT HIV-1 RT 15-fold more tightly than CTP even though both have 2' OH group that clash with the Y115 residue. Possibly, the absence of a 3'-OH in 3'dCTP creates a space that allows the 2'-OH to avoid the steric hindrance of Y115 by repositioning itself during the entry to the binding pocket. When the hydroxyl group is absent (Y115F mutant), the binding affinity of 3'dCTP to Y115 RT is almost as high as the canonical dCTP to WT RT. This bolsters the hypothesis that both the loss of the hydroxyl group in tyrosine in the Y115F mutant and the absence of a 3'-OH in 3'dCTP allows a non-canonical substrate to bind as tightly as the canonical dCTP in a mutant enzyme.

The rNTP incorporation of HIV-1 RT occurs because of the dearth of canonical dNTPs in non-dividing macrophages. This irregular event is restricted in T cells due to the abundance of dNTPs (2,13). The host SAMHD1 protein, which hydrolyzes and depletes dNTPs, is responsible for the low dNTP concentrations observed in macrophages. Unlike HIV-1, HIV-2 and many SIV strains, such as SIV sooty mangabey encode Vpx. Vpx targets SAMHD1 for proteosomal degradation upon infection and elevates cellular dNTP concentrations above the K_m value of HIV-1 RT (3,8,9). Therefore, it is a reasonable assumption that unlike HIV-1, HIV-2 and SIVsm strains do not incorporate rNTPs even in non-dividing macrophages. It is also plausible that the rNMPs incorporated in (–) strand DNA, which induces pausing of HIV-1 RT, significantly delays the (+) strand DNA synthesis kinetics in macrophages. However, the (+) strand DNA synthesis should not be delayed in HIV-2 and SIVsm because Vpx elevates dNTP concentrations, which restricts the rNMP incorporation in the (–) strand DNA template (3,8). Similarly, the rNTP incorporation-induced events such as the delayed (+) strand DNA synthesis do not occur in activated CD4⁺ T cells due to an abundance of dNTPs in an actively dividing cell type (3,4).

4.1 Conclusions

The data presented in this study reveal the mechanism of rNTP incorporation by HIV-1 RT and the kinetic effect of how embedded rNMPs in the DNA template can influence reverse transcription rates. However, two questions about rNTP incorporation events in non-dividing macrophages remain unanswered: 1) whether the rNMPs incorporated into proviral DNA are repaired in the cells, and 2) whether the proviral DNA containing rNMP affects viral transcription by host RNA polymerase II after its integration into the host chromosomal DNA. Understanding these rNTP incorporation-induced molecular events will reveal the unique nature of HIV-1 infection in non-dividing myeloid cell types, which may serve as HIV-1 reservoirs.

Acknowledgments

We would like to thank Dr. Joseph Hollenbaugh and Gina Lenzi for critical comments about this manuscript. This study was supported by NIH AI049781 (B.K.), GM104198 (B.K.), and 5P30-AI-50409 Centers for AIDS Research and the Department of Veterans Affairs.

ABBREVIATIONS

rNTP	ribonucleotide
rNMP	ribonucleoside monophosphate
dNMP	deoxyribonucleoside monophosphate
dNTP	deoxyribonucleotide
HIV-1	Human Immunodeficiency Virus 1
RT	HIV-1 reverse transcriptase
SIV	Simian Immunodeficiency Virus
HIV-2	Human Immunodeficiency Virus-2
T/P	template/primer
3'dCTP	3'-deoxycytidine-5'-triphosphate
SAMHD1	sterile alpha motif and HD domain 1
Vpx	viral protein X

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Highlights

- Pre-steady state kinetic analysis shows HIV-1 RT rNTPs incorporation rate was slower than when dNTPs was substrate.
- HIV-1 RT selectivity for dNTP versus rNTP is lowest with ATP, so it incorporates ATP more readily under low dATP conditions
- dNTP incorporation before an embedded rNMP resulted in a higher K_d and a slower k_{pol} than a template containing dNMP
- HIV-1 RT Y115F mutant has a lower discrimination against rNTPs due to a lower K_d

FIGURE 1. HIV-1 wild type RT active site determination and rNTP incorporation

(A) Representative curve of pre-steady state kinetics of dTTP incorporation. HIV-1 RT was preincubated with a combination of 100 nM [γ -³²P] ATP 5' labeled "Extend T" primer annealed to a 38-mer RNA template and 200 nM of cold unlabeled T/P. Single nucleotide extension was measured by adding 800 μM of dTTP to each reaction at the indicated time points (seconds). Using equation 1, the burst shows 80% active HXB2 wild type (WT) RT protein. (B) Representative K_d curves are shown for AMP incorporation using 10 to 5000 μM concentrations. The observed rate was plotted using equation 2. A representative ATP kinetics curve (open circles) shows a K_d of 66.14 μM and the k_{pol} was 17.12 s⁻¹. (C) A representative GTP (open triangles) curve shows the K_d of 248.4 μM and the k_{pol} was 0.92

s^{-1} . A representative CTP kinetics curve (open diamonds) shows the K_d was 1482 μM and the k_{pol} was 0.25 s^{-1} . A representative UTP kinetics curve (open squares) shows the K_d was 831 μM and the k_{pol} was 0.19 s^{-1} .

FIGURE 2. Diagram of the DNA template containing an embedded ribonucleoside

A diagram of the DNA templates used in our kinetics experiment along with the respective primers which are labeled as 21-mer P, 22-mer P, and 23-mer P. The DNA template contains an embedded dA, which is our control template is labeled as the “dA Template”. The DNA template containing an embedded rA is labeled as the “rA Template”. The primers were individually annealed to either the dA or rA template, which allowed pre-steady state kinetic analysis of single nucleotide incorporation across from one of the respective positions -1, 0, or +1 on the embedded rA or dA template. The pre-steady state kinetics data of single nucleotide incorporation at these specified positions are shown in Table 2.

FIGURE 3. HIV-1 RT WT and Y115F mutant incorporation of 3'dCTP drug

(A) An illustration of different nucleotide substrate dCTP, CTP and 3'dCTP drug utilized in our HIV-1 WT and Y115F pre-steady state kinetic analysis comparison. From left to right showing dCTP lacking the 2' OH group, CTP containing 2' and 3' OH groups, and 3'dCTP analog lacking the 3' OH group. (B) A comparison of K_d curves from WT HIV-1 RT and Y115F RT utilizing 3'dCTP drug. Y115F RT has a similar k_{pol} to WT RT (k_{pol} 0.09 ± 0.01 and k_{pol} 0.05 ± 0.01 respectively). The Y115F RT has a K_d ($18.60 \pm 1.02 \mu\text{M}$) that is 6-fold lower for 3'dCTP than WT RT (K_d 112.30 ± 15.70), which makes Y115F RT's catalytic efficiency rate for 3'dCTP incorporation 12-fold higher than WT RT. (C) A comparison of selectivity ($(k_{pol}/K_d)_{dCTP}/(k_{pol}/K_d)_{3'dCTP}$) for WT RT and Y115F RT using values from Table 3 is displayed. WT RT selectivity value for dCTP vs. 3'dCTP is 16805 ± 2653.5 and Y115F RT selectivity value is 1714 ± 297.4 .

Table 1

Pre-steady state kinetic constants for rNTP and dNTP incorporation using wild type HIV-1 RT.

Nucleotides (rNTP/dNTP)	$k_{\text{pol}} \text{ (s}^{-1}\text{)}^a$	$K_d \text{ (}\mu\text{M)}^a$	$k_{\text{pol}}/K_d \text{ (}\mu\text{M}^{-1}\text{s}^{-1}\text{)}^a$	Selectivity a, b
UTP	0.20 ± 0.01	989.50 ± 158.50	$2.02 \times 10^{-4} \pm 3.39 \times 10^{-5}$	39702 ± 9443.9
TTP	310.10 ± 11.11	38.69 ± 2.02	8.02 ± 0.51	
CTP	0.25 ± 0.00	1645.40 ± 162.70	$1.52 \times 10^{-4} \pm 1.51 \times 10^{-5}$	16805 ± 2653.5
dCTP	96.00 ± 0.12	38.2 ± 2.31	2.51 ± 0.15	
GTP	0.92 ± 0.11	248.40 ± 32.09	$3.70 \times 10^{-3} \pm 6.52 \times 10^{-4}$	565 ± 136
dGTP	89.04 ± 1.48	42.50 ± 2.28	2.09 ± 0.12	
ATP	17.12 ± 2.77	66.14 ± 10.50	0.259 ± 0.06	20 ± 5.7
dATP	248.40 ± 4.49	48.54 ± 1.83	5.12 ± 0.21	

^aValues represent mean $\pm$ standard deviation from two independent experiments.

^bDefined as $(k_{\text{pol}}/K_d)_{\text{dNTP}}/(k_{\text{pol}}/K_d)_{\text{rNTP}}$.

Table 2

Kinetics of dNTP incorporation with a template containing an embedded ribonucleoside.

Nucleotides Positions	Template	k_{pol} (s ⁻¹) ^a	K_d (μM) ^a	k_{pol}/K_d (μM ⁻¹ s ⁻¹) ^a
dATP Position -1	dA	38.05 ± 0.26	1.77 ± 0.28	21.50 ± 3.34
21-mer	rA	1.86 ± 0.08	50.74 ± 4.77	0.04 ± 0.00
dTTP Position 0	dA	21.99 ± 1.07	1.43 ± 0.19	15.38 ± 1.32
22-mer	rA	91.50 ± 5.50	13.70 ± 1.27	6.68 ± 0.22
dGTP Position +1	dA	34.85 ± 1.93	1.52 ± 0.44	24.62 ± 5.86
23-mer	rA	22.21 ± 1.47	0.56 ± 0.15	42.03 ± 8.68

^aValues represent mean ± standard deviation from two independent experiments.

Table 3

Pre-steady state kinetic parameters of Y115F HIV-1 RT proteins with three nucleotides containing different sugar moieties.

Nucleotide Incorporation	RT	k_{pol} ($\text{s}^{-1}\mu\text{M}$) ^a	K_d (μM) ^a	k_{pol}/K_d ($\mu\text{M}^{-1}\text{s}^{-1}$) ^a	Selectivity ^{a,b}
dCTP	Y115F	52.90 ± 1.04	15.20 ± 0.19	3.47 ± 0.08	1.38 ± 0.116
CTP	Y115F	0.26 ± 0.01	125.40 ± 17.78	$2.03 \times 10^{-3} \pm 2.99 \times 10^{-4}$	13.7 ± 3.33
3'dCTP	WT Y115F	0.05 ± 0.01 0.09 ± 0.01	112.30 ± 15.70 18.60 ± 1.02	$4.28 \times 10^{-4} \pm 8.51 \times 10^{-5}$ $5.07 \times 10^{-3} \pm 4.55 \times 10^{-4}$	12.5 ± 3.56

^aValues represent mean ± standard deviation from two independent experiments.

^bDefined as $Y115F\text{ RT } (k_{\text{pol}}/K_d)_{\text{nucleotide}}/\text{WT RT } (k_{\text{pol}}/K_d)_{\text{nucleotide}}$