Human Immunodeficiency Virus Type-1 Reverse Transcriptase: Contribution of Met-184 to Binding of Nucleoside 5'-Triphosphate*

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Mutations were made in recombinant human immunodeficiency virus type-1 reverse transcriptase (RT) by substituting methionine 184 with alanine (M184A) or valine (M184V), and steady-state and pre-steady-state kinetic constants were determined. The $K_m$ values of M184A RT for dNTPs were larger than those of wt RT for RNA-directed synthesis; the $k_{cat}$ values of M184A RT for processes or distributive synthesis were similar. In contrast to M184A RT, the $K_m$ and $k_{cat}$ values of M184V RT for dNTP substrates were similar to those of wt RT. The $K_m$ values of M184V RT for 1-$beta$-nucleoside analogs were increased 30-500-fold relative to wt RT for both RNA- and DNA-directed synthesis. The $K_m$ and $k_{cat}$ values of wt RT and M184V RT for dCTP and cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-y]cytosine 5'-triphosphate (1-$beta$-FTCTP) were estimated from pre-steady-state kinetics for single nucleotide incorporation. The $K_m$ value of M184V RT for 1-$beta$-FTCTP was 30-fold greater than that of wt RT; the $k_{cat}$ values of the two enzymes were similar. These results support the hypothesis that methionine 184 in the highly conserved YMDD region of wt RT participates in the binding of the nucleoside (analog) 5'-triphosphate.

The YXDD region is a highly conserved sequence in RNA and DNA polymerases and reverse transcriptases (1). This region is postulated to be part of the active site for the polymerase domain in plant, animal, and bacterial viral polymerases (1-5). Dependent upon the polymerase, the tyrosine and two aspartic acids flank a hydrophobic amino acid (X) (1, 6). In human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT), this hydrophobic amino acid residue is a methionine. Evidence from numerous workers implicates this as a critical region in the polymerization domain of RT (7-11). RTs with substitutions at the tyrosine or two aspartic acid sites show decreased polymerase activity, while the RNase H activity remains unaffected (7, 9). Furthermore, Lowe et al. (10) demonstrated that mutations at the tyrosine and two aspartic acids of RT increase the $K_m$ value for dNTP substrate. Data from several viral replication studies also suggest that this region is important in catalysis (8, 11). HIV-1 with a substitution of serine or alanine for Met-184 replicates slower that wild-type virus, while virus containing a substitution of glycine or proline for Met-184 cannot replicate (11). In addition, Larder et al. (8) demonstrated that virus containing a substitution of serine for tyrosine at position 185 (Y185S) replicates slowly.

Several groups have reported that HIV-1 with a substitution in the RT domain at Met-184 to valine (M184V HIV-1) is resistant to several nucleoside analogs (12-15). This mutant virus was first isolated by in vitro selection for virus resistant to ddl; furthermore, M184V HIV-1 is also cross-resistant to ddC (12). The level of resistance, however, is low. The IC$_{50}$ values of M184V HIV-1 for ddl and ddC are 5-fold larger than those for wild type HIV-1. A more significant effect is seen on the IC$_{50}$ values of M184V HIV-1 for the 1-$beta$-l-enantiomer of FTC. The IC$_{50}$ value of M184V HIV-1 for 1-$beta$-L-FTCTP is >5000-fold larger than that of wt HIV-1 (13, 14). These results suggest that HIV-1 can tolerate a substitution in this highly conserved motif that significantly affects the IC$_{50}$ value for 1-$beta$-L-FTCTP without affecting the replication of the virus (11).

Our laboratory is interested both in the mechanism of decreased viral replication rates caused by mutations at position 184 of RT and in the mechanism of drug resistance with respect to ddl, ddC, and FTC. We conducted steady-state and pre-steady-state kinetic analyses of wt RT, M184A RT, and M184V RT for both natural dNTPs and chain-terminating 1-$beta$- and 1-$beta$-l-nucleotide analogs. The results demonstrate that the decreased viral replication rates observed with M184A HIV-1 and the increase in IC$_{50}$ values for nucleoside analogs observed with M184V HIV-1 correlate with an increase in the $K_m$ value of HIV-1 RT for nucleoside 5'-triphosphate.

EXPERIMENTAL PROCEDURES

Kinetic Model for HIV-1 RT—A simplified kinetic model for HIV-1 RT was reported previously (16). This model assumes that the chemical step and the conformational step have been combined into a single step. CBVTP, carbovir 5'-triphosphate; FTCTP, cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-y]cytosine 5'-triphosphate; 3TCTP, 1-$beta$-2-deoxy-3-thiacytidine 5'-triphosphate; BCH$_{189}$ 5'-triphosphate, 1-$beta$-2-deoxy-3-thiacytidine 5'-triphosphate; 1-$beta$-FddCTP, 1-$beta$-5 fluoro-2'-3'-dideoxycytidine 5'-triphosphate; ddC, 2',3'-dideoxycytidine; ddL, 2',3'-dideoxyinosine.

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1 The abbreviations used are: HIV-1, human immunodeficiency virus type-1; RT, reverse transcriptase; dNTP, 2'-deoxyribonucleoside 5'-triphosphates; wt, wild-type; FTC, cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-y]cytosine; AZTTP, 3'-azido-3'-deoxythymidine 5'-triphosphate; dATTP, 3'-deoxy-2',3'-dideoxythymidine 5'-triphosphate; (Received for publication, January 4, 1996, and in revised form, April 1, 1996)
kinetic step identified as $k_{f}$, the concentration of template-primer is much greater than its dissociation constant for free enzyme; and the kinetic constants for translation of the enzyme on E-T7, binding of dNTP, and dissociation of PoP, are large relative to $k_{p}$. Processive synthesis is defined as DNA synthesis in which multiple nucleotides are incorporated into the primer by the processive primer to dissociation of E-T7 ($k_{p} \ll k_{f}$) (Scheme 1), where $k_{p}$ is the template-primer $T_{p}$ complex. The M13mp18HXBRT is the template-primer after incorporation of one nucleotide, and dNTP is the nucleoside $5^{'}-$ triphosphate. Under these conditions, $K_m = k_{p}$ and $k_{f} = k_{p}$. Distinctive synthesis occurs when the E-T7 $T_{p}$ complex dissociates after each nucleotide addition (Scheme 2). During nonprocessive synthesis, $k_{f} > k_{p}$, $K_m = k_{f}$, and $k_{p} = k_{f}$. During forced termination of processive synthesis, as is the case with incorporation of a chain-terminating nucleotide analog, $k_{f} < k_{p}$, $K_m = k_{{p}(k_{f})}$, and $k_{p} = k_{f}$.

Materials—[2532P]ATP, [H]dATP, [H]dCTP, [H]dTTP, and [H]dTTP were purchased from DuPont NEN. dNTPs, 2',3'-dideoxyribonucleotides, poly(A)(dT)$_{10}$, poly(C)(dG)$_{12}$, poly(rI), and di(pG)pol(rC) were obtained from Pharmacia Biotech Inc. The 5'-GTTAGT TGATGTG $T_{23}$-mer was purchased from Pharmacia Biotech Inc. The Ready-To-Go™ polynucleotide kinase kit used to add kinase $\alpha$-32P-label to the d23-mer was purchased from Pharmacia Biotech Inc. The Ready-To-Go™ large-scale T7 transcription kit. The sequences of the template and primer are as follows. 7.75 mM Tris-HCl (pH 7.8), was kindly provided by Wayne Miller (Burroughs Wellcome Co.).

Tributylammonium phosphate was obtained from Sigma. All other chemicals were of analytical grade or better. wt RT, M184V RT, and M184A RT were cloned, expressed, and purified as described by White et al. (21), except that 1,3-dimethyl-3,4,5,6-tetrahydropyrimidine was used as a co-solvent. The solution was removed from the reaction mixture by extraction with chloroform, the nucleoside $5^{'}-$ triphosphate was purified by ion-exchange chromatography. The products were absorbed onto a Pharmacia Mono Q 10/10 anion-exchange column equilibrated in 0 M ammonium bicarbonate, and the nucleoside analog 5'-triphosphate was eluted with a linear gradient of ammonium bicarbonate from 0 to 700 M ammonium bicarbonate in a single peak, resulting in a 34% yield; 3TCTP and BCH189 5'-triphosphate were prepared as described by White et al. (22), except that 1,3-dimethyl-3,4,5,6-tetrahydropyrimidine was used as a co-solvent. 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Met-184 of HIV-1 RT Contributes to Nucleotide Binding

TABLE I.

Km and kcat values of wt RT, M184V RT, and M184A RT for dNTPs

| dNTP      | wt RT | M184V RT | M184A RT |
|------------|-------|----------|----------|
| dATP      |       |          |          |
| dCTP      |       |          |          |
| dGTP      |       |          |          |
| dTTP      |       |          |          |
| RNA-directed |     |          |          |
| dATP      | 0.08 ± 0.01 | 0.09 ± 0.03 | ND* |
| dCTP      | 6.0 ± 0.5 | 10 ± 1 | 80 ± 20 |
| dGTP      | 2.8 ± 0.7 | 4.7 ± 1.6 | ND |
| dTTP      | 2.4 ± 0.6 | 4 ± 1 | 120 ± 20 |
| DNA-directed |     |          |          |
| dATP      | 0.79 ± 0.06 | 1.0 ± 0.5 | ND |
| dCTP      | 2.6 ± 1.2 | 3.0 ± 0.7 | 2.9 ± 0.5 |
| dGTP      | 0.7 ± 0.3 | 0.5 ± 0.4 | ND |
| kcat      | 1.6 ± 0.1 | 3.5 ± 0.4 | 3.4 ± 0.6 |

* a r44·d23-mer was used for dATP turnover, poly(r1)·(dC)19–24 for dCTP turnover, poly(rC)·(dG)12–18 for dGTP turnover, and poly(rA)·(dT)10 for dTTP turnover.
ND, not determined.

The template-primer used for all dNTPs for DNA-directed synthesis was pM13mp18HXBR T as described under "Experimental Procedures.”

with 25 μM KCl. Reactions with poly(r1)·(dC)19–24 contained Buffer A with 2 mM MgCl2, instead of 5 mM (higher MgCl2 concentrations caused precipitation of the template-primer). Reactions with r44·d23-mer and r44·d24-mer contained Buffer A with 50 mM KCl. All assays were initiated with enzyme with the exception of those with poly(r1)·(dC)19–24, which were initiated with the template primer.

Presteady-state Kinetic Assays—Presteady-state kinetic data were collected as described previously by Reardon (24) using a rapid-mixing apparatus purchased from Kin-Tec (25). Briefly, the enzyme-labeled template-primer complex in 50 mM Tris-HCl (pH 7.8), 50 mM KCl, 2.5 mM EDTA, and 0.025% Triton X-100 (Buffer B) was mixed at 25 °C with varying concentrations of substrate in Buffer B with 15 mM MgCl2. The reactions were quenched with 0.5 mM EDTA at selected times between 0.03 and 10 s after initiation of the reaction. Reactions to determine the kinetic parameters of wt RT for dCTP and 1-β-L-FTCTP contained 200 nM enzyme, 10 mM r44·(dC)23-mer, and substrate at selected concentrations ranging from 1 to 20 μM. Reactions to determine the kinetic parameters of M184V RT for 1-β-L-FTCTP contained 400 nM enzyme, 10 mM r44·(dC)23-mer, and 1-β-L-FTCTP at selected concentrations ranging from 400 to 3 μM. The quenched samples (100 μl) were desalted by two Bio-Rad Biospin columns. The desalted samples were lyophilized; resuspended in a solution of 45% formamide, 0.025% xylene cyanol FF, and 0.025% bromphenol blue; heated at 90 °C for 5 min; and rapidly cooled on ice. The products were separated by electrophoresis on an 8% urea, 15% polyacrylamide gel, and the DNA was quantitated by phosphorimager and the software ImageQuant. Equation 1 was fitted to the time courses for product formation, where A is the amplitude of the burst, Kp is the observed first-order rate constant, [S] is the nucleoside (analogue) 5′-triphosphate concentration, and t is time. Five to six concentrations of substrate were used to determine the nucleoside (analogue) 5′-triphosphate dissociation constant (Kd) and burst rate constant (kcat).

[Product] = A(1 - e^(-Kp[S]/Kd[S]))t (Eq. 1)

RESULTS

Steady-state Kinetics for Nucleotides—The Km and kcat values of wt RT, M184V RT, and M184A RT were determined for the natural dNTPs. The kcat values of M184V RT and M184A RT were not significantly different from those of wt RT for RNA- and DNA-directed DNA synthesis, indicating that neither of the mutations affected the rate-limiting step of the reaction (Table I). The kcat is equal to the kcat for RT-catalyzed nucleotide incorporation into the homopolymeric template-primers poly(r1)·(dC)19–24, poly(rC)·(dG)12–18, and poly(rA)·(dT)10, which were utilized to determine the kinetic constants for dCTP, dGTP, and dTTP, respectively. The kcat values of wt RT and M184V RT for dATP incorporation for RNA-directed synthesis were significantly smaller than those for dCTP, dGTP, and dTTP incorporation. In this case, RT catalyzed the incorporation of dAMP into the defined sequence template-primer r44·d23-mer, where the only nucleotide present was the next encoded nucleotide, dATP; therefore, the enzyme was forced to dissociate from the template primer after each dAMP incorporation. Under these circumstances of forced termination of processive synthesis, kcat is equal to koff for E-TPr1–z.

The Km values of M184V RT for the natural dNTPs during either RNA- or DNA-directed DNA synthesis were not different from those of wt RT, suggesting that the mutation to valine did not significantly affect natural substrate binding (Table I). In addition, the Km values of M184A RT for dCTP and dTTP for DNA-directed synthesis were similar to those of wt RT. In contrast to RNA-directed synthesis, the Km values of M184A RT for dCTP and dTTP for RNA-directed synthesis were 14- and 70-fold larger than those of wt RT, respectively. Therefore, the Km values of M184A RT were larger than those of wt RT specifically for RNA-directed synthesis. To determine if this difference between RNA- and DNA-directed synthesis found for M184A RT was due to the template-primers used (homopolymeric template-primers were used for RNA-directed synthesis, and the heteropolymeric template-primer pM13mp18HXBR T was used for DNA-directed synthesis), Km values were determined using the template-primers r44·d23-mer and d45·d29-mer under conditions of forced termination in which the first nucleotide to be incorporated into each template-primer was dCTP. For r44·d23-mer, the Km value for dCTP of wt RT was 21 nM, and that of M184A RT was 800 nM; the substitution of alanine for Met-184 resulted in a 40-fold larger Km value for RNA-directed synthesis. With d45·d29-mer as the template-primer, the Km value for dCTP of wt RT was 200 nM, and that of M184A RT was 320 nM; substitution of alanine for Met-184 did not significantly affect the Km value for dCTP. Thus, the Km value of M184A RT was significantly larger than that of wt RT for RNA-directed synthesis, but not for DNA-directed synthesis.

Steady-state Kinetics for Nucleotide Analogs—The K values of wt RT and M184V RT for 1-β-L- and 1-β-D-nucleotide analogs were determined for both RNA- and DNA-directed DNA synthesis (Table II). The K values for both 1-β-D- and 1-β-L-enantiomers of cytidine, guanosine, and thymidine nucleotide analogs were determined to evaluate enantiomeric selectivity of M184V RT.

The K values of wt RT for inhibitors of RNA-directed synthesis varied from 3 nM to 2 μM. This is not a reflection that, for example, ddGTP (with a K value of 4.2 nM) binds to RT better than ddCTP (with a K value of 770 nM). As noted previously, these differences were due to the fact that different template-primers were used for each nucleotide bases assayed. Therefore, only the K values determined using the same individual template-primer can be compared meaningfully.

The K values of M184V RT for ddATP (the active metabolite of dideoxyinosine) and ddCTP for RNA-directed synthesis were
than those of wt RT (32–110-fold). Therefore, M184V RT had 2–3-fold larger than those of wt RT. In addition, the
for dTTP turnover.

azidodeoxycytidine 1-

value of M184ART for dCTP was 14-fold larger than that of

The template-primer used for all dNTPs for DNA-directed synthesis was pM13mp18HXBRT as described under “Experimental Procedures.”

RNA-directed synthesis

| Substrate analog | Enantiomer | Ki<sub>wt RT</sub> (µM) | Ki<sub>M184V RT</sub> (µM) | Ki<sub>M184V RT</sub>/Ki<sub>wt RT</sub> |
|------------------|------------|-----------------|-----------------|-----------------|
| ddATP           | 1-β-D      | 0.11 ± 0.02     | 0.7 ± 0.02      | 6 ± 1           |
| ddCTP           | 1-β-D      | 0.8 ± 0.2       | 2.4 ± 0.6       | 3 ± 1           |
| (−)-ddCTP       | 1-β-L      | 1.7 ± 0.1       | 130 ± 40        | 80 ± 30         |
| (+)-BCH189      | 1-β-D      | 1.2 ± 0.1       | 4.8 ± 0.2       | 4 ± 0.4         |
| 3TCTP           | 1-β-L      | 0.97 ± 0.05     | 80 ± 30         | 80 ± 30         |
| (+)-FTCTP       | 1-β-D      | 1.0 ± 0.1       | 5.4 ± 0.3       | 5.4 ± 0.6       |
| (−)-FTCTP       | 1-β-L      | 0.60 ± 0.01     | 190 ± 10        | 320 ± 10        |
| ddGTP           | 1-β-D      | 0.004 ± 0.002   | 0.006 ± 0.004   | 1.5 ± 0.8       |
| CBVTP           | 1-β-L      | 0.0095 ± 0.0008 | 0.004 ± 0.001   | 0.15 ± 1.0      |
| (+)-CBVTP       | 1-β-L      | 0.010 ± 0.0010  | 0.03 ± 0.07     | 30 ± 3         |
| ddTTP           | 1-β-D      | 0.0032 ± 0.0002 | 0.0081 ± 0.0007 | 2.5 ± 0.2       |
| (−)-ddTTP       | 1-β-L      | 0.11 ± 0.03     | 8.8 ± 0.1       | 80 ± 20        |
| d4TTP           | 1-β-L      | 0.0052 ± 0.0002 | 0.020 ± 0.004   | 3.8 ± 0.2       |
| (−)-d4TTP       | 1-β-L      | 0.015 ± 0.004   | 1.6 ± 0.5       | 110 ± 30       |
| AZTTP           | 1-β-D      | 0.0050 ± 0.0006 | 0.0040 ± 0.0008 | 0.8 ± 0.1       |

DNA-directed synthesis

| Substrate analog | Enantiomer | Ki<sub>wt RT</sub> (µM) | Ki<sub>M184V RT</sub> (µM) | Ki<sub>M184V RT</sub>/Ki<sub>wt RT</sub> |
|------------------|------------|-----------------|-----------------|-----------------|
| ddATP           | 1-β-D      | 0.19 ± 0.04     | 0.20 ± 0.09     | 1.1 ± 0.3       |
| ddCTP           | 1-β-D      | 1.3 ± 0.2       | 1.4 ± 0.3       | 1.1 ± 0.3       |
| (−)-ddCTP       | 1-β-L      | 2.2 ± 0.2       | 60 ± 10         | 27 ± 7         |
| (+)-BCH189      | 1-β-D      | 0.53 ± 0.02     | 0.90 ± 0.03     | 2 ± 1          |
| 3TCTP           | 1-β-L      | 1.7 ± 0.2       | 36 ± 8          | 50 ± 20        |
| (+)-FTCTP       | 1-β-L      | 0.44 ± 0.01     | 0.41 ± 0.04     | 0.9 ± 0.6      |
| (−)-FTCTP       | 1-β-L      | 0.43 ± 0.06     | >200             | >500        |
| ddGTP           | 1-β-D      | 0.15 ± 0.04     | 0.10 ± 0.02     | 0.7 ± 0.2       |
| CBVTP           | 1-β-D      | ND              | ND              | ND            |
| (+)-CBVTP       | 1-β-L      | ND              | ND              | ND            |
| ddTTP           | 1-β-D      | 0.069 ± 0.002   | 0.28 ± 0.19     | 4.1 ± 1.4      |
| (−)-ddTTP       | 1-β-L      | 1.1 ± 0.1       | 28 ± 0.8        | 26 ± 2        |
| d4TTP           | 1-β-D      | 0.08 ± 0.02     | 0.08 ± 0.01     | 1.0 ± 0.3       |
| (−)-d4TTP       | 1-β-L      | 0.13 ± 0.02     | 7.2 ± 1.3       | 55 ± 9      |
| AZTTP           | 1-β-D      | 0.044 ± 0.002   | 0.07 ± 0.02     | 1.6 ± 0.7       |

2–3-fold larger than those of wt RT. In addition, the Ki values for the cytidine analogs BCH189 5′-triphosphate and 1-β-D-FTCTP were 4- and 5-fold larger, respectively, and the Ki values for ddTTP and d4TTP were 2- and 4-fold larger, respectively, than those of wt RT. No differences in the Ki values of wt RT and M184V RT were observed for the 1-β-D-guanosine analogs ddGTP and CBVTP and the 1-β-L-thymidine analog AZTTP. In addition, no differences were observed in the Ki values of wt RT and M184V RT for 1-β-D-nucleoside analogs for DNA-directed synthesis. Larger differences were observed between the Ki values of wt RT and M184V RT for nucleoside analogs of the 1-β-L-conformation for both RNA- and DNA-directed DNA synthesis. The Ki values of M184V RT for the 1-β-L-cytidine analogs were significantly larger than those of wt RT (80–320-fold). Additionally, the Ki values of M184V RT for 1-β-L-CBVTP, ddTTP, and d4TTP were significantly larger than those of wt RT (32-110-fold). Therefore, M184V RT had larger Ki values relative to wt RT for all 1-β-L-nucleoside analogs studied for both RNA- and DNA-directed DNA synthesis, regardless of base or sugar substitutions. The Ki values of M184A RT, M184V RT, and wt RT for the 5′-triphosphates of ddC, 1-β-L-ddC, BCH189, 3TCT, and 1-β-D- and 1-β-L-FTCTP are summarized in Table III. The Ki values of M184A RT for 1-β-D-ddC, 1-β-L-ddC, BCH189, 3TCT, and 1-β-D- and 1-β-L-FTCTP were increased by 5- and 3-fold relative to wt RT, respectively. The Ki values of M184A RT for the thia cytidine analogs were increased 40-, 30-, 30-, and 85-fold relative to wt RT for BCH189 5′-triphosphate, 3TCTP, 1-β-D- and 1-β-L-FTCTP, respectively. Because the Ki value of M184A RT for dCTP was 14-fold larger than that of wt RT, it is more appropriate to compare differences seen in the Ki/Km ratios of wt RT and M184A RT to determine if utilization of the nucleoside analogs has been more significantly compromised than dCTP utilization. When these values were analyzed, no significant differences were observed (Table III). In fact, the Ki/Km ratios of M184A RT for 1-β-D- and 1-β-L-ddCTP were lower than those found for wt RT. These data suggest the Ki values of M184A RT for all nucleotide (analog) substrates, regardless of stereochemistry, have been affected to approximately the same extent by the substitution of Met-184 with alanine.

1-β-L-Cytidine Analogs as Alternate Substrates—1-β-D- and 1-β-L-ddCTP, 3TCTP, and FTCTP were assayed to determine if the 1-β-L-compounds were substrates of M184V RT (Fig. 1). The 1-β-D- and 1-β-L-enantiomers of ddCTP, FTCTP, and 3TCTP were all incorporated into r44[32P]dT23-mer by M184V RT and were therefore substrates for the enzyme. Pre-steady-state Kinetics for wt RT and M184V RT—The equilibrium dissociation constant (K<sub>d</sub>) and the burst rate constant (k<sub>b</sub>) of wt RT and M184V RT for dCTP and 1-β-L-FTCTP were calculated from the concentration dependence of the pseudo-first-order rate constant for dNMP incorporation into template-primer (Equation 1). The experimental conditions were such that, after the first round of incorporation of substrate in the burst phase of the reaction, r44[32P]dT23-mer was exhausted, and no steady-state rate of substrate incorporation was observed. Fig. 2 shows these data for wt RT and M184V RT with 1-β-L-FTCTP as the substrate. These data are not shown for dCTP. For wt RT, the dissociation constant for dCTP was 16...
Met-184 of HIV-1 RT Contributes to Nucleotide Binding

The template-primer used was poly(l)·(dc)_{18–20}. 

\[ K_v \] values for wt RT, M184V RT, and M184A RT for cytidine analogs

| Substrate | wt RT | M184V RT | M184A RT |
|-----------|-------|----------|----------|
| ddCTP | 0.8 ± 0.2 | 2.4 ± 0.6 | 4 ± 2 |
| 1-β-β-ddCTP | 1.7 ± 0.1 | 12.0 ± 4.0 | 5.1 ± 1 |
| 1-β-β-BCH189 | 1.2 ± 0.1 | 4.8 ± 0.2 | 49 ± 6 |
| 3TCTP | 0.97 ± 0.05 | 80 ± 30 | 29 ± 6 |
| 1-β-β-FTCTP | 1.0 ± 0.1 | 5.4 ± 0.3 | 31 ± 4 |
| 1-β-β-FTCTP | 0.60 ± 0.01 | 190 ± 10 | 52 ± 6 |

\[ K_v(\text{wt RT}) \] was significantly lower than that of \[ K_v(M184V) \] (Table II).

**DISCUSSION**

The YMDD motif spanning amino acids 183–186 of HIV-1 RT has been reported to comprise part of the putative active site of the enzyme (26, 27). A substitution of alanine at position 184 in this highly conserved region significantly compromised the replication rate of the resulting mutant virus, whereas a substitution of valine had no effect (11). The 

\[ K_v \] values of both mutant enzymes for dTTP turnover with poly(rA)·(dT)_{10} and for ddCTP turnover with poly(rA)·(ddC)_{19–24} were similar to those determined for wt RT. HIV-1 RT catalyzes processive synthesis with poly(rA)·(dT)_{10} as the template-primer and nonprocessive synthesis with poly(rA)·(dc)_{19–24} as the template-primer; 

\[ k_{cat} \] is equal to \[ k_p \] for both types of synthesis, based on the simplified kinetic model. In addition, \[ K_v \] values of M184A RT and M184V RT for dNTP binding to E-TP_{n+1}. These data indicate that substitution of valine or alanine for Met-184 did not affect \[ k_p \] or \[ K_v(\text{M184A}) \] for E-TP_{n+1}. The \[ k_{cat} \] values for M184V RT for dNTP synthesis were also determined under conditions of forced termination with the template r44-mer and primers d24-mer and d23-mer, respectively; these values were similar to those determined for wt RT. In the case of forced termination of processive synthesis, \[ k_{cat} \] is equal to \[ k_p \] for E-TP_{n+1}. These data indicate that substitution of valine or alanine for Met-184 did not affect \[ k_p \] or \[ K_v(\text{M184A}) \] for E-TP_{n+1}. The \[ K_v \] values for M184A RT were larger than those of wt RT for processive synthesis (poly(rA)·(dT)_{10}), nonprocessive synthesis (poly(rA)·(dc)_{19–24}), and forced termination of processive synthesis (r44·d23-mer). The \[ K_v(\text{M184A}) \] value for substrate during processive and nonprocessive synthesis is equal to \[ k_{cat} \] for E-TP_{n+1}. The \[ K_v \] value for substrate during forced termination of processive synthesis is equal to \[ k_{cat}(\text{M184A})/k_p(\text{M184A}) \]. Because the values of \[ k_{eff} \] and \[ k_p(\text{M184A}) \] were similar to those of wt RT, the equilibrium binding constant of M184A RT for dNTP has been affected by the substitution at Met-184. No significant effect was observed on the \[ K_v \] values of M184V RT for dNTPs during processive or nonprocessive synthesis with poly(rA)·(dT)_{10} as the template-primer and nonprocessive synthesis with poly(rA)·(dc)_{19–24} as the template-primer; 

The relationship between the burst rate constant and the steady-state kinetic parameters is determined by the ratio of 

\[ k_{cat} \] values for wt RT, M184V RT, and M184A RT for dNTP has been affected by the substitution at Met-184. No significant effect was observed on the \[ K_v \] values of M184V RT for dNTPs during processive or nonprocessive synthesis with poly(rA)·(dT)_{10} as the template-primer and nonprocessive synthesis with poly(rA)·(dc)_{19–24} as the template-primer; 

\[ k_{cat} \] is equal to \[ k_p \] for both types of synthesis, based on the simplified kinetic model. In addition, \[ K_v \] values of M184A RT and M184V RT for dNTP binding to E-TP_{n+1}. These data indicate that substitution of valine or alanine for Met-184 did not affect \[ k_p \] or \[ K_v(\text{M184A}) \] for E-TP_{n+1}. The \[ k_{cat} \] values for M184V RT for dNTP synthesis were also determined under conditions of forced termination with the template r44-mer and primers d24-mer and d23-mer, respectively; these values were similar to those determined for wt RT. In the case of forced termination of processive synthesis, \[ k_{cat} \] is equal to \[ k_p \] for E-TP_{n+1}. These data indicate that substitution of valine or alanine for Met-184 did not affect \[ k_p \] or \[ K_v(\text{M184A}) \] for E-TP_{n+1}. The \[ K_v \] values for M184A RT were larger than those of wt RT for processive synthesis (poly(rA)·(dT)_{10}), nonprocessive synthesis (poly(rA)·(dc)_{19–24}), and forced termination of processive synthesis (r44·d23-mer). The \[ K_v(\text{M184A}) \] value for substrate during processive and nonprocessive synthesis is equal to \[ k_{cat} \] for E-TP_{n+1}. The \[ K_v \] value for substrate during forced termination of processive synthesis is equal to \[ k_{cat}(\text{M184A})/k_p(\text{M184A}) \]. Because the values of \[ k_{eff} \] and \[ k_p(\text{M184A}) \] were similar to those of wt RT, the equilibrium binding constant of M184A RT for dNTP has been affected by the substitution at Met-184. No significant effect was observed on the \[ K_v \] values of M184V RT for dNTPs during processive or nonprocessive synthesis with poly(rA)·(dT)_{10} as the template-primer and nonprocessive synthesis with poly(rA)·(dc)_{19–24} as the template-primer; 

\[ k_{cat} \] is equal to \[ k_p \] for both types of synthesis, based on the simplified kinetic model. In addition, \[ K_v \] values of M184A RT and M184V RT for dNTP binding to E-TP_{n+1}. These data indicate that substitution of valine or alanine for Met-184 did not affect \[ k_p \] or \[ K_v(\text{M184A}) \] for E-TP_{n+1}. The \[ k_{cat} \] values for M184V RT for dNTP synthesis were also determined under conditions of forced termination with the template r44-mer and primers d24-mer and d23-mer, respectively; these values were similar to those determined for wt RT. In the case of forced termination of processive synthesis, \[ k_{cat} \] is equal to \[ k_p \] for E-TP_{n+1}. These data indicate that substitution of valine or alanine for Met-184 did not affect \[ k_p \] or \[ K_v(\text{M184A}) \] for E-TP_{n+1}. The \[ K_v \] values for M184A RT were larger than those of wt RT for processive synthesis (poly(rA)·(dT)_{10}), nonprocessive synthesis (poly(rA)·(dc)_{19–24}), and forced termination of processive synthesis (r44·d23-mer). The \[ K_v(\text{M184A}) \] value for substrate during processive and nonprocessive synthesis is equal to \[ k_{cat} \] for E-TP_{n+1}. The \[ K_v \] value for substrate during forced termination of processive synthesis is equal to \[ k_{cat}(\text{M184A})/k_p(\text{M184A}) \]. Because the values of \[ k_{eff} \] and \[ k_p(\text{M184A}) \] were similar to those of wt RT, the equilibrium binding constant of M184A RT for dNTP has been affected by the substitution at Met-184. No significant effect was observed on the \[ K_v \] values of M184V RT for dNTPs during processive or nonprocessive synthesis with poly(rA)·(dT)_{10} as the template-primer and nonprocessive synthesis with poly(rA)·(dc)_{19–24} as the template-primer;
thesis or forced termination of processive synthesis. Thus, the catalytic efficiency of M184A RT (k_{cat}/K_m) is less than that of M184V RT and wt RT. Therefore, the slower replication rates of M184A HIV-1 relative to wt HIV-1 and M184V HIV-1 in cell culture are probably due to an increase in the K_m value for dNTP binding to E:TP_m, possibly allowing reverse transcription to become rate-limiting in the replication life cycle of M184A HIV-1.

Interestingly, the K_m values of M184A RT for dNTPs are larger than those of wt RT only for RNA-directed DNA synthesis. Similarly, the K_i values of M184V RT for ddATP and ddCTP (the active metabolites of dideoxyinosine and dideoxycytidine, respectively) were larger (6- and 3-fold, respectively) than those of wt RT only for RNA-directed DNA synthesis. These results indicate that Met-184, in conjunction with the template-primer, contributes to the binding energy for nucleotide substrates such that mutations specifically affect binding of a 1-β-D-nucleotide to an HIV-1 RT-RNA-DNA complex. A DNA-DNA helix usually adopts a B-form configuration free in solution (28, 29). However, when HIV-1 RT is co-crystallized with a double-stranded DNA template-primer, the DNA adopts an A-form configuration in the polymerase domain (30). The configuration of an RNA-DNA template-primer complexed with HIV-1 RT is unknown. The data presented here suggest that the configuration of an RNA-DNA helix complexed with HIV-1 RT differs from that of a DNA-DNA helix complexed with HIV-1 RT.

HIV-1 containing the M184V substitution is not only resistant to dideoxyinosine and dideoxycytidine, but is also resistant to the 1-β-L-thiacytidine analogs 1-β-L-FTCTP and 3TC (13–15). Faraj et al. (20) demonstrated that the K_i values of M184V RT for 1-β-L-ddCTP, 1-β-L-ddCTP, and 3TC with poly(dI:dC) 10–15 as the template-primer are all significantly larger than the wt RT values, correlating with the significant increases (>2000-fold) in the I_{50} values of M184V HIV-1 for 1-β-L-FTCTP and 3TC.

We used steady-state and pre-steady-state analyses to determine if RT containing the substitution at position 184 to valine had elevated K_m values with respect to wt RT for 1-β-L-cytidine analogs exclusively or 1-β-L-nucleoside analogs in general. To begin to address these questions, a series of 1-β-L-nucleoside analogs were studied: three pairs of cytidine analogs (which included thiacytidine analogs), two pairs of thymidine analogs, and one pair of guanosine analogs. The K_i values of M184RT for all 1-β-L-nucleoside analogs determined were larger than those of wt RT. Interestingly, the effect of the valine substitution on the K_i values for 1-β-L-nucleoside analogs was not limited to RNA-directed DNA synthesis, as was observed with M184A RT for dNTPs and M184V RT for 1-β-D-2',3'-dideoxynucleotides. The data obtained with 1-β-L-nucleoside analogs indicate that the M184V mutation allows the reverse transcriptase to become highly enantioselective and suggest that virus containing this mutation would be resistant to any 1-β-L-nucleoside analog, regardless of substitutions in the sugar or base portions of the nucleoside. In contrast, the selectivity of M184A RT suggests that virus containing this mutation would not be enantioselective.

1-β-L-Nucleoside analogs are alternate substrates for wt RT (17, 20, 31). To address this question with regard to M184V RT, an incorporation experiment was performed in which 1-β-D- and 1-β-L-ddCTP, 3TCTP, and FTCTP were incubated with wild-type and mutant enzymes bound to r44[^2P]d23-mer for a fixed time. All three 1-β-L-cytidine analogs were substrates for the mutant enzyme; however, it would appear from this experiment that 1-β-L-ddCTP is not as good a substrate for M184V RT compared with 3TCTP and 1-β-L-FTCTP, even though the K_i values determined under steady-state conditions were similar. The decreased substrate efficiency of 1-β-L-ddCTP relative to 3TCTP and 1-β-L-FTCTP could be the result of an increased K_m decreased K_{cat}, or a combination of both of these parameters. In contrast to our results, Faraj et al. (20) have reported that 1-β-L-ddCTP is not a substrate for M184V RT. This difference is most likely due to the differences in concentration of 1-β-L-ddCTP used in each study. In the study reported by Faraj et al., the concentration of 1-β-L-cytidine analogs in both the wt

![Fig. 2. Concentration dependence of the time course of 1-β-L-FTCTP incorporation into DNA by wt RT and M184V RT. A, wt RT(r44[^2P]d23-mer) was mixed with 1-β-L-FTCTP at 50 μM (●), 25 μM (▲), 10 μM (■), 5 μM (▲), and 2.5 μM (●). B, M184V RT(r44[^2P]d23-mer) was mixed with 1-β-L-FTCTP at 400 μM (●), 150 μM (▲), 50 μM (■), 30 μM (▲), 10 μM (■), and 3 μM (●).](image-url)

| Table IV | Kinetic constants determined by pre-steady-state kinetics |
|---------------------------------|---------------------------------|
| Enzyme | Substrate | k_d | K_m | K_{cat} | K_{cal} |
| wt RT | dCTP | 2 | 16 | 0.07 | 0.015 |
| M184V RT | 1-β-L-FTCTP | 0.24 | 1.7 | 0.13 | 0.06 |
| | 1-β-L-FTCTP | 0.18 | 40 | 0.049 | 0.04 |

*a K_m values for dCTP were determined at 25°C using r44·d23-mer as the template-primer. K_{cat} values reported for 1-β-L-FTCTP were determined by Dixon plot analysis competing against dCTP turnover using r44·d23-mer as the template-primer.

b K_{cal} = k_{eff}/k_{cat} for forced termination of processive synthesis, where k_{eff} = k_{cat} = 0.0083 s⁻¹.
RT and M184V RT assays was 1 μM; the $K_d$ value of M184V RT was 60-fold larger than that of wt RT (120 and 2.0 μM, respectively). In the assay reported here, the concentration of the 1-$\beta$-l-cytidine analogs in the mutant RT assays was 20-fold higher than in the wt RT assays. If $k_{\text{cat}}$ values were similar for wt RT and M184V RT, then under the assay conditions of Faraj et al., M184V RT would have 1.7% of the activity of wt RT for 1-$\beta$-l-ddCTP turnover, which, in the 15-min time course of the assay, could account for the observed lack of 1-$\beta$-l-ddCTP turnover with M184V RT.

Steady-state data for dCTP and 1-$\beta$-l-FTCTP indicated that the two substrates had similar $K_m$ values for wt RT; with r44d23-mer as the template-primer, the $K_m$ value for dCTP was 70 nM, and that for 1-$\beta$-l-FTCTP was 130 nM. However, pre-steady-state kinetic analysis demonstrated that, even though the steady-state $K_m$ values were similar, the $K_d$ and $k_p$ values for the two substrates were very different. The $K_d$ value for dCTP (16 μM) was 10-fold higher than that for 1-$\beta$-l-FTCTP (1.7 μM); therefore, 1-$\beta$-l-FTCTP is a tighter binding substrate than dCTP. The observed first-order rate constant ($k_f$) for dCTP was 9 s$^{-1}$, and that for 1-$\beta$-l-FTCTP was 0.24 s$^{-1}$, yielding $k_f/K_d$ values of 0.56 and 0.14 s$^{-2}$ μM$^{-1}$, respectively. The $K_m$ value for substrate during forced termination of pro-cessive synthesis is $k_{\text{off}}/(K_d/K_p)$; the $K_m$ values of wt RT for dCTP and 1-$\beta$-l-FTCTP were similar because the respective $k_p/K_m$ ratios were similar.

Steady-state data for M184A RT suggested that a mutation at this position affected the $K_d$ value for nucleotide substrate. This was confirmed using pre-steady-state kinetic analysis; the mutation to valine at Met-184 resulted in a 2-fold increase in $K_d$ for dCTP and a 20-fold increase in $K_d$ for 1-$\beta$-l-FTCTP. Therefore, the increase in I$_{50}$ values of HIV-1 M184V for 1-$\beta$-l-nucleoside analogs correlates with a significant increase in the equilibrium binding constant constantly for the 1-$\beta$-l-substrate. However, when comparing the $K_d$ values of M184V RT for dCTP and 1-$\beta$-l-FTCTP, it was not evident as to why the $K_m$ value for 1-$\beta$-l-FTCTP was higher than that for dCTP; the mutant enzyme bound 1-$\beta$-l-FTCTP as well as dCTP. It was only clear when the $k_p$ values for the two compounds were compared. Turnover of substrate by M184V RT into the growing strand of DNA was 50-fold slower for 1-$\beta$-l-FTCTP than for dCTP, resulting in $k_f/K_d$ ratios of M184V RT for dCTP of 0.23 s$^{-1}$ μM$^{-1}$ and for 1-$\beta$-l-FTCTP of 0.0056 s$^{-1}$ μM$^{-1}$. Thus, 1-$\beta$-l-FTCTP was a less efficient substrate than dCTP of M184V RT because the $k_p/K_m$ ratio was 40-fold lower.

The data presented in this study are further evidence that the YMDD domain is important in catalysis for HIV-1 RT. Specifically, the amino acid at position 184 in the YMDD loop directly participates in nucleotide substrate binding.