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# Exploring the Effects of Active Site Constraints on HIV-1 Reverse Transcriptase DNA Polymerase Fidelity*

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**To examine the concept of polymerase active site tightness as a criteria for DNA polymerase fidelity, we performed pre-steady-state single nucleotide incorporation kinetic analyses with sugar modified thymidine 5′-triphosphate (TTP) analogues and human immunodeficiency virus (HIV-1) reverse transcriptase (RT). The employed TTP analogues ($T^RTP$) are modified at the 4′-position of the sugar moiety with alkyl groups, gradually expanding their steric demand. Introduction of a methyl group reduces the maximum rate of nucleotide incorporation by about 200-fold for $RT^{WT}$ and about 400-fold for $RT^{M184V}$. Interestingly, the affinity of RT for the modified nucleotide is only marginally affected. Increasing the size to an ethyl group leads to further reduction of the rate of incorporation and first effects on binding affinities are observed. Finally, substitution for an isopropyl group results not only in a further reduction of incorporation rates but also in a dramatic loss of binding affinity for the nucleotide analogue. By increasing the steric demand the effects on $RT^{M184V}$ in comparison with $RT^{WT}$ become progressively more pronounced. Misincorporation of either TTP or $T^{Me}TP$ opposite a template G causes additional decline in incorporation rates accompanied by a drastic decrease in binding affinities. This results in relative incorporation efficiencies $[(k_{pol}/K_d)_{incorrect}/(k_{pol}/K_d)_{TTPcorrect}]$ of $4.1 \times 10^{-5}$ for TTP and $3.4 \times 10^{-6}$ for $T^{Me}TP$ in case of $RT^{WT}$ and $1.4 \times 10^{-5}$ for TTP and $2.9 \times 10^{-8}$ for $T^{Me}TP$ in case of $RT^{M184V}$.**

The intrinsic error frequencies of DNA polymerases are typically in the range of $10^{-3}$ to $10^{-5}$ per base replicated (1–3). Most eukaryotic DNA polymerases show higher fidelity, whereas virally encoded polymerases are more error-prone. Recently, a new class of so called bypass polymerases have been discovered (4–8). These polymerases are thought to have certain functions in DNA repair rather than replication and show error rates as high as 1 per 22 bases (9). Thus, depending on their function, polymerases show different degrees of selectivity for the nucleotide substrate. For example, it is conceivable that a viral polymerase has lower fidelity than a cellular polymerase, enabling the virus to escape the host immune system response by an increased mutation rate. Although our knowledge about polymerases has grown substantially in the past few years, the mechanistic details for this varied substrate selectivity are not fully understood (10–12). It is generally accepted that Watson-Crick hydrogen bonding by itself does not account for the observed selectivity. Several additional factors have been discussed to be involved in correct nucleotide recognition (2, 13–15). Among these factors are exclusion of water from the active site of the enzyme, base stacking, solvation, minor groove scanning, and steric constraints within the nucleotide binding pocket (16). It remains to be determined to what extent these factors contribute to polymerase fidelity.

Here we used the human immunodeficiency virus (HIV)[1] reverse transcriptase (RT) as a model system to examine the concept of active site tightness and substrate fit as a major determinant of nucleotide selectivity. The HIV-1 enzyme shows a moderate fidelity of about $10^{-4}$ (17, 18). Interestingly, the mutation M184V, which provides high level resistance to the drug Lamivudine (3TC), has been shown to result in increased fidelity (19–22). Structural investigations indicate that a $\beta$-methyl side chain present in valine contacts the sugar ring of the incoming triphosphate, leading to steric hindrance (23–25). This might indicate that small changes of the geometry of the nucleotide binding pocket indeed affect fidelity. If this is the case, modifications of the nucleotide at this position should be felt by the enzyme leading to enhanced discrimination. As a steric probe, we used sugar modified thymidine 5′-triphosphate (TTP) analogues (26). In these TTP analogues ($T^RTP$) the 4′-hydrogen position of the sugar is substituted with alkyl groups (-$CH_3$, -$CH_2CH_3$, and -$CH(CH_3)_2$), gradually expanding their steric demand.

Recently, we demonstrated that these compounds are well tolerated by the Klenow fragment of *Escherichia coli* DNA polymerase I. Their added size apparently increased selectivity, strongly supporting the steric model (26, 27). Furthermore, we reported on functional investigations of HIV-1 RT employing these size-augmented analogues $T^RTP$ (28). Performing steady-state kinetic analyses, we found little difference between both enzymes when promoting "correct" incorporation of the different $T^RTP$s. However, in misincorporation events the two enzymes behave differently. While 4′-methylation had little effect on the selectivity of HIV-1 RT, significant effects were observed for the Klenow fragment. Thus, these results may be a first evidence in support of the concept of active site tightness as a causative effect of differential fidelities among DNA polymerases.

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[1] The abbreviations used are: HIV, human immunodeficiency virus; RT, reverse transcriptase; $T^RTP$, thymidine 5′-triphosphate (R = -H, -$CH_3$, -$CH_2CH_3$, and -$CH(CH_3)_2$); WT, wild type; p/t, primer/template; dNTP, deoxynucleoside triphosphate.

While steady-state kinetic analysis provides useful insights into the process of polymerase fidelity (29, 30), this method only detects the rate-limiting step of the overall polymerase cycle, which is the dissociation of RT from the extended p/t. Accordingly, this technique is not capable of elucidating protein/nucleotide interactions at the active site during nucleotide incorporation. DNA synthesis by RT follows an ordered reaction pathway (31–33). The first step is the binding of the nucleic acid substrate resulting in the formation of a tight RT·p/t complex in the low nanomolar range. Now the deoxynucleoside triphosphate (dNTP) enters the active site and binds in a two-step process (34). In a fist step a loose complex is formed. This is followed by a conformational change in the enzyme (*e.g.* closure of the fingers domain), leading to the formation of a tight ternary complex. The second step in dNTP binding represents the rate-limiting step for nucleotide incorporation and has been proposed to be responsible for the correct positioning of the dNTP within the binding pocket and accordingly determines specificity (35). The ternary complex then catalyzes the nucleophilic attack of the 3′-hydroxyl of primer on the α-phosphate of the dNTP resulting in nucleotide incorporation. Subsequently, pyrophosphate is released, and the enzyme either dissociates from the p/t (distributive mode) or translocates along the template to incorporate the next nucleotide (processive mode). The incorporation of the dNTP is thus defined by three kinetic steps: initial loose nucleotide binding, the rate-limiting induced fit, and the actual rapid chemical step.

In this study we report about pre-steady-state kinetic measurements analyzing the correct and incorrect incorporation of 4′-modified nucleotides in comparison with the natural counterpart by HIV-1 RT wild-type and mutant M184V. This enables us to differentiate between certain steps during the polymerase pathway. Our data clearly show that the induced fit leading to a tight ternary complex is the main determinant of nucleotide selectivity. In addition, binding effects come into play when the steric distortion reaches a certain limit. Thus, our results support the idea that steric constraints within the nucleotide binding pocket are of major importance for polymerase fidelity.

## EXPERIMENTAL PROCEDURES

*Proteins*—Recombinant heterodimeric wild-type and M184V mutant HIV-1 RTs were expressed in *E. coli* and purified as described before (36, 37). Enzyme concentrations were routinely determined using an extinction coefficient at 280 nm of 260,450 $M^{-1}$ $cm^{-1}$.

*Buffers*—All experiments were carried out at 25 °C in a buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 50 mM KCl. Annealing buffer consisted of 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl.

*Modified Thymidine 5′-Triphosphates*—4′-Modified thymidine 5′-triphosphates ($T^RTP$) were synthesized as described previously (26).

*Oligonucleotides*—Oligodeoxynucleotides were purchased from a commercial supplier and purified by denaturing polyacrylamide gel electrophoresis (15% acrylamide, 7 M urea) followed by elution from the gel using a Schleicher & Schuell Biotrap unit.

The sequence of the 24/36-mer DNA/DNA p/t was 5′-GTGGTGCGAAATTTCTGACAGACA and 5′-GTGCGTCTGTCX TGTCTGTCAGAAATTCTGCACCAC (*X* = A for correct insertion; *X* = G for misinsertion), respectively. Primer oligodeoxynucleotides were 5′-end-labeled using T4 polynucleotide kinase as described (37). Primer and template oligodeoxynucleotides were annealed by heating equimolar amounts in annealing buffer at 90 °C, followed by cooling to room temperature over several hours in a heating block. The completeness of the reaction was checked by determining whether 100% of the primer of the hybridized and radioactively labeled p/t could be extended by one nucleotide. The samples were analyzed on 10% denaturing gels.

*Rapid Kinetics of Nucleotide Incorporation*—Rapid-quench experiments were carried out in a chemical quench-flow apparatus (RQF-3, KinTek Corp., University Park, PA). Reactions were started by rapidly mixing the two reactants (15 μl of each) and then quenched with 0.6% trifluoroacetic acid at defined time intervals. All concentrations reported are final concentrations after mixing in the rapid-quench apparatus. Products were analyzed by denaturing gel electrophoresis (10% polyacrylamide/7 M urea) and quantitated by scanning the dried gel using a phosphorimager (Fuji FLA 5000). Data were evaluated using the program Grafit (Erithacus Software).

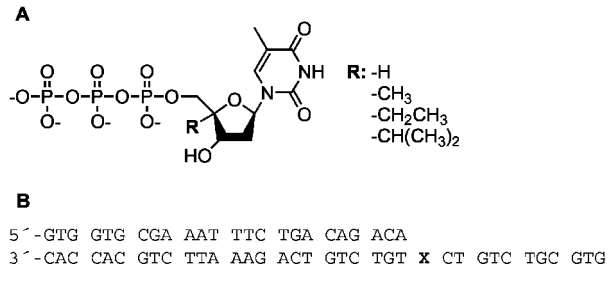


FIG. 1. **Substrates used in this study.** Steric probes $T^RTP$ (*A*) and sequence of the 24/36 primer/template (*B*) are shown.

For pre-steady-state kinetics, a preformed complex of p/t·RT (100 nM p/t and 200 nM RT) was rapidly mixed with an excess of dNTP (100 μM to 4 mM) and stopped after various times in the millisecond to second range. Data were fitted to a burst equation (single or double exponential plus a linear equation). The effective pre-steady-state constants ($k_{pol}$) at the given dNTP concentration were derived from the exponential rates.

Affinities of $T^RTP$s were determined by the dependence of the pre-steady-state burst rate on the $T^RTP$s concentration. To measure the affinities of the $T^RTP$s the preformed p/t·RT (100 and 200 nM) complex was rapidly mixed with various concentrations of $T^RTP$s and quenched after $t_{½}$ of the maximal pre-steady-state rate. The corresponding rates were then calculated from the concentration of elongated primer by converting the exponential equation into $k = -\ln[1 - ([P_{+1}]_t/[P]_0)]/t(s)$. $[P]_0$ corresponds to the concentration of RT·p/t complex available for incorporation at $t = 0$ (burst amplitude), and $t$ equals the reaction time ($t_{½}$ of the maximal pre-steady-state rate). The observed rates were plotted against the $T^RTP$ concentration, and the dissociation constant ($K_d$) was calculated by fitting the data to a hyperbola.

*Misincorporation Kinetics*—Misincorporation experiments were performed manually. Reactions were started by mixing equal volumes (5 μl) of the two reactants and then stopped with 0.6% trifluoroacetic acid after defined time intervals. Products were analyzed as described above. Dissociation constants were determined as described in the previous section using $T^RTP$ concentrations in the range of 1 μM to 6 mM.

## RESULTS

*Time Course of Single Turnover, Single Nucleotide Incorporation*—In a first set of experiments we analyzed single turnover, single nucleotide incorporation kinetics of $T^RTP$ nucleotides into a 24/36 DNA/DNA p/t substrate by $RT^{WT}$ and $RT^{M184V}$, respectively. All experiments were carried out under saturating concentrations of p/t and nucleotide. To ensure that the single turnover rate of incorporation observed is limited by internal rate-limiting kinetic parameters, rather than by binding parameters, which occurs when concentrations are used below the saturation level, we carefully examined binding affinities of the incoming dNTP (see section below).

Fig. 1 shows the structure of the different analogues and the sequence of the p/t used in this study. Incorporation of $T^HTP$ by the two enzymes showed a biphasic burst of product formation followed by a slower linear phase (Fig. 2). The linear, steady-state phase was shown to be caused by the rate-limiting dissociation of the extended p/t product from the enzyme (33). In agreement with earlier findings, we observed complex kinetics as indicated by the two burst phases (33, 38, 39). The first, fast phase corresponds to a productive enzyme-substrate complex which is capable of nucleotide incorporation. The second, slower phase represents a nonproductive complex, which has to undergo an isomerization before dNTP incorporation can occur. This phenomenon has been described in detail recently (33). The amplitude of the first burst phase is somewhat smaller

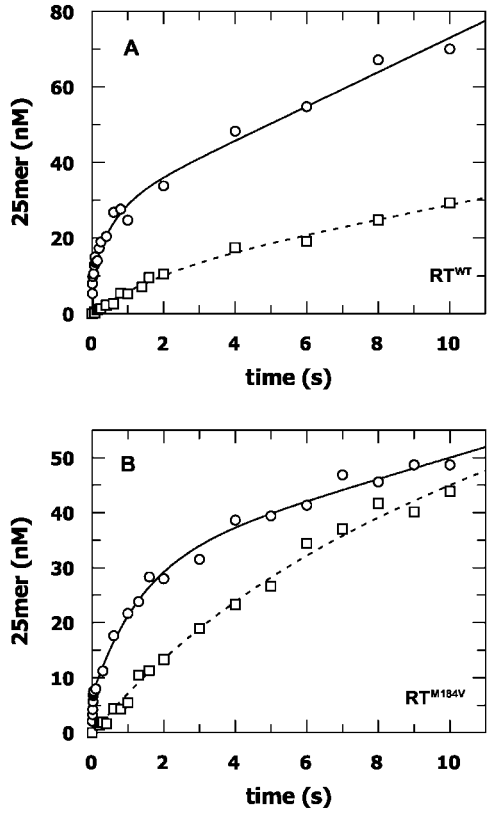


FIG. 2. **Single turnover, single $T^{H}TP$, or $-T^{Me}TP$ incorporation into 24/36 DNA/DNA p/t by HIV-1 $RT^{WT}$ and $RT^{M184V}$.** The curves show the best fit of the data to a double or single exponential equation plus slope. A preformed complex of 200 nM $RT^{WT}$ (*A*) or $RT^{M184V}$ (*B*) and 100 nM p/t was rapidly mixed with either 100 $\mu$M $T^{H}TP$ (○) or 100 $\mu$M $T^{Me}TP$ (□). The exponential analysis of the data for $RT^{WT}$ yielded two burst rates ($k_{pol1}$ and $k_{pol2}$) of 92.6 $s^{-1}$ (± 36.6) and 1.6 $s^{-1}$ (± 0.6) for $T^{H}TP$ (*solid line*, double exponential) and a burst rate of 0.5 $s^{-1}$ (± 0.17) for $T^{Me}TP$ (*dashed line*, single exponential). The analysis of the experimental data for $RT^{M184V}$ gave burst rates of 74.0 $s^{-1}$ (± 27.9) and 0.7 $s^{-1}$ (± 0.1) for $T^{H}TP$ (*solid line*, double exponential) and 0.18 $s^{-1}$ (± 0.03) for $T^{Me}TP$ (*dashed line*, single exponential).

than observed previously. This difference can be attributed to different primer length used in these studies.[2] Fitting of the experimental data to a double exponential equation plus slope yielded rates ($k_{pol1}$ and $k_{pol2}$) of 92.6 $s^{-1}$ (± 36.6) and 1.6 $s^{-1}$ (± 0.6) for $RT^{WT}$ and 74 $s^{-1}$ (± 28) and 0.7 $s^{-1}$ (± 0.1) for $RT^{M184V}$ (results are summarized in Table I).

Upon incorporation of $T^{Me}TP$ burst rates dropped dramatically for both enzymes (Fig. 2). Fitting of the experimental data to a single exponential equation plus slope yielded rates ($k_{pol1}$) of 0.5 $s^{-1}$ (± 0.17) and 0.18 $s^{-1}$ (± 0.03) for $RT^{WT}$ and $RT^{M184V}$, respectively. Increasing the size of the substitution at the 4′-position of the sugar resulted in further decrease of nucleotide incorporation rates. The corresponding rates for the incorporation of $T^{Et}TP$ and $T^{iPr}TP$ are listed in Table I.

*$T^{R}TP$ Binding Affinity for Correct Nucleotide Insertion*—As outlined above, actual kinetic constants can only be derived from single turnover experiments when substrate concentrations are not limiting. We therefore examined the binding affinity of both enzymes for each $T^{R}TP$ nucleotide used in this study. Consequently, the rate dependence on concentration for $T^{R}TP$ with p/t-bound RT was determined by plotting the observed rates at various concentrations of $T^{R}TP$ and fitting the data to a hyperbolic curve (Fig. 3). The best fit to the hyperbolic equation relating the rate of incorporation to the nucleotide concentration yielded $T^{H}TP$ dissociation constants ($K_d$ values) of 11.7 $\mu$M (± 1.4) and 5.5 $\mu$M (± 1.0) for $RT^{WT}$ and $RT^{M184V}$, respectively, consistent with previous measurements (21, 37). Analysis of the binding affinities for $T^{Me}TP$ resulted in $K_d$ values of 19.0 $\mu$M (± 1.6) and 16.1 $\mu$M (± 3.1) for $RT^{WT}$ and $RT^{M184V}$, respectively. As anticipated, by further increasing the size of the sugar modification by introducing an ethyl or isopropyl group, the $K_d$ values for these analogues decline gradually with constants of 15.2 $\mu$M (± 2.3) and 45.2 $\mu$M (± 3.5) for $T^{Et}TP$ and 314.5 $\mu$M (± 28.2) and 1001 $\mu$M (± 106) for $T^{iPr}TP$ ($RT^{WT}$ *versus* $RT^{M184V}$).

*Single Turnover Nucleotide Misincorporation of $T^{R}TP$ Opposite Template G*—To gain insights whether size expansion by 4′-alkylation has an impact on fidelity of nucleotide insertion, we performed single turnover nucleotide misincorporation of $T^{R}TP$ opposite template G with both RTs. As described above all experiments were set up to be performed under saturating nucleotide concentrations. However, due to substrate inhibition the maximal reasonable $T^{R}TP$ concentration was in the range of about 6 mM. Since the observed incorporation rates were too slow to be measured with the quench apparatus, experiments were conducted using manual quenching methods.

Fig. 4 shows the time courses of misincorporation of $T^{H}TP$ and $T^{Me}TP$ by either $RT^{WT}$ or $RT^{M184V}$. The curves show the best fit to a single exponential equation plus slope. For $RT^{WT}$ we determined incorporation rates of 0.07 $s^{-1}$ (± 0.0056) and 0.03 $s^{-1}$ (± 0.0023) for $T^{H}TP$ and $T^{Me}TP$, respectively. The mutant enzyme showed even lower rates of 0.1 $s^{-1}$ (± 0.01) and 0.002 $s^{-1}$ (± 0.0002) for $T^{H}TP$ and $T^{Me}TP$. Comparing the relative DNA-dependent DNA replication fidelity of both RTs calculated as $[((k_{pol}/K_d)_{TTP})_{incorrect}/((k_{pol}/K_d)_{analogue})_{incorrect}]$, the wild-type enzyme shows 12-fold lower misincorporation probability of $T^{Me}TP$ *versus* $T^{H}TP$, whereas the mutant enzyme shows a 488-fold lower likelihood. In other words, $RT^{M184V}$ is about 40 times more sensitive toward misincorporation of modified *versus* unmodified nucleotide.

*$T^{R}TP$ Binding Affinity for Incorrect Nucleotide Insertion*—Analogous to the experiments described above, we determined nucleotide binding affinities in the situation of non-Watson-Crick base pairing (*e.g.* incoming T opposite template G). The best fit to a hyperbolic equation relating the rate of misincorporation to the nucleotide concentration yielded a $T^{H}TP$ dissociation constants ($K_d$) of 208.1 $\mu$M (± 14) and 512.6 $\mu$M (± 26.6) for $RT^{WT}$ and $RT^{M184V}$, respectively (Fig. 5). For $T^{Me}TP$ we could derive $K_d$ values of 1089.5 $\mu$M (± 58.2) and > 5000 $\mu$M for $RT^{WT}$ and $RT^{M184V}$, respectively. Due to substrate inhibition above 6 mM nucleotide, we can only give a lower limit of 5 mM for the $K_d$ of $T^{Me}TP$-$RT^{M184V}$·p/t interaction.

## DISCUSSION

In this study we have examined the effect of steric nucleotide probes ($T^{R}TP$) on DNA polymerase fidelity of HIV-1 RT. If the concept of active site tightness being a major factor for polymerase selectivity holds true, such probes should have marked effects on incorporation fidelity. The underlying principle of this approach is rather straightforward. By increasing the size of a given nucleotide by 4′-alkylation, it will less likely be accepted by the polymerase due to steric constraints within the nucleotide binding pocket. In addition, analyzing RT carrying the M184V mutation, which has been proposed to cause steric hindrance within the active site, this effect should be even more pronounced.

We found that incorporation of $T^{H}TP$ by both enzymes, $RT^{WT}$ and $RT^{M184V}$, showed very similar incorporation kinetics as well as binding affinities for the nucleotide. This finding was not surprising and has been reported earlier (21, 37). Addition-

[2] T. Restle, unpublished observation.

TABLE I
*Kinetic and equilibrium constants for binding and incorporation/misincorporation of $T^RTP$ by HIV-1 $RT^{WT}$ and $RT^{M184V}$*

| $T^RTP$ | $K_d$ $T^RTP$ | | $k_{pol}$ | | Incorporation efficiency (i.e.)[a] | | Relative i.e. | Selectivity factor[b] | |
|---|---|---|---|---|---|---|---|---|---|
| | $RT^{WT}$ | $RT^{M184V}$ | $RT^{WT}$ | $RT^{M184V}$ | $RT^{WT}$ | $RT^{M184V}$ | $RT^{WT}/RT^{M184V}$ | $RT^{WT}$ | $RT^{M184V}$ |
| | $\mu M$ | | $s^{-1}$ | | | | | | |
| Opposite A: | | | | | | | | | |
| H | 11.7 ± 1.4 | 5.5 ± 1.0 | 92.6 ± 36.6 | 74.0 ± 27.9 | 1 | 1 | | | |
| Me | 19.0 ± 1.6 | 16.1 ± 3.1 | 0.5 ± 0.17 | 0.18 ± 0.03 | $3.3 \times 10^{-3}$ | $8.3 \times 10^{-4}$ | 3.9 | 308 | 1244 |
| Et | 15.2 ± 2.3 | 45.2 ± 3.5 | 0.15 ± 0.01 | 0.06 ± 0.005 | $1.2 \times 10^{-3}$ | $9.8 \times 10^{-5}$ | 12.2 | 810 | 10547 |
| iPr | 314.5 ± 28.2 | 1001.1 ± 106.7 | 0.03 ± 0.005 | 0.004 ± 0.0003 | $1.2 \times 10^{-5}$ | $2.9 \times 10^{-7}$ | 41 | 83,867 | 3,503,850 |
| Opposite G: | | | | | | | | | |
| H | 208.1 ± 14.0 | 512.6 ± 26.6 | 0.07 ± 0.0056 | 0.1 ± 0.01 | $4.1 \times 10^{-5}$ | $1.4 \times 10^{-5}$ | 2.9 | | |
| Me | 1089.5 ± 58.2 | >5000[c] | 0.03 ± 0.0023 | 0.002 ± 0.0002 | $3.4 \times 10^{-6}$ | $2.9 \times 10^{-8}$ | >117 | | |

[a] i.e., incorporation efficiency $[(k_{pol}/K_d)T^RTP]/RT^{WT\text{-or-}M184V}[(k_{pol}/K_d)T^{H}TP]$.
[b] Calculated as $[(k_{pol}/K_d)_{TTP}/(k_{pol}/K_d)_{analogue}]$.
[c] Not measured under saturating nucleotide concentrations due to substrate inhibition.




FIG. 3. **Dependence of the pre-steady-state burst rate on $T^{H}TP$ (○) and $T^{Me}TP$ (□) concentration.** Increasing amounts of $T^RTP$ were rapidly mixed with a preformed complex of either 200 nM $RT^{WT}$ (*A*) or $RT^{M184V}$ (*B*) and 100 nM p/t. Reactions were quenched after $t_{½}$ of the maximal pre-steady-state rate (see "Experimental Procedures"). Data were fitted to a hyperbolic equation, yielding $K_d$ values for $RT^{WT}$ of 11.7 $\mu$M (± 1.4) for $T^{H}TP$ and 19.0 $\mu$M (± 1.6) for $T^{Me}TP$, and for $RT^{M184V}$ of 5.5 $\mu$M (± 1.0) for $T^{H}TP$ and 16.1 $\mu$M (± 3.1) for $T^{Me}TP$.

ally, this proves that the M184V mutation has no effect on the DNA polymerase activity of RT. On the other hand, $RT^{M184V}$ has been reported to confer enhanced fidelity (19–22). This has been attributed due to steric constraints of the $\beta$-methyl side chain present in valine that is believed to contact the sugar ring of the incoming triphosphate (23–25). To our surprise, introduction of a rather small methyl group at the 4′-position of the sugar ring, led to an ~200-fold reduction of the pre-steady-state $RT^{WT}$ nucleotide incorporation rate, without affecting binding affinities. For the $RT^{M184V}$ this effect is even more pronounced, yielding an ~400-fold reduction. This suggests that the rate-limiting step for nucleotide incorporation, the induced fit (*e.g.* closure of the fingers), is affected. Exchanging methyl for ethyl at the 4′-position results in further reduction of the incorporation rate combined with a slight decrease in binding affinity in case of the mutant enzyme. Finally, the $T^{iPr}TP$ analogue shows the most dramatic effect with incorporation as well as binding being affected. It seems that the initial nucleotide binding step tolerates modifications up to the size of an ethyl group, and selection takes place during the second step of nucleotide binding. In all cases the mutant enzyme incorporates the $T^RTP$ analogues with significant lower efficiency. There is a 4-fold decrease in $RT^{M184V}$ efficiency compared with $RT^{WT}$ for $T^{Me}TP$, 13-fold for $T^{Et}TP$, and 41-fold for $T^{iPr}TP$ (see Table I for details). This finding clearly supports the idea that the valine instead of the methionine within the polymerase active site causes steric hindrance, thus monitoring the size augmentation of the nucleotide probe. As a result incorporation efficiency decreases. In addition, this ver-

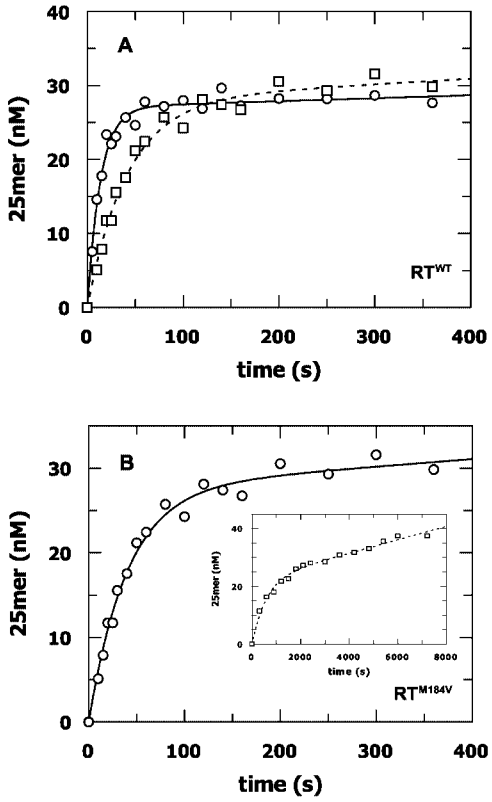


FIG. 4. **Single turnover kinetics of misincorporation of $T^{H}TP$ and $T^{Me}TP$ opposite a template G into 24/36 DNA/DNA p/t by HIV-1 $RT^{WT}$ and $RT^{M184V}$.** Preformed complexes of 200 nM enzyme and 100 nM p/t were rapidly mixed with $T^{H}TP$ (○) or $T^{Me}TP$ (□) and quenched at the time points indicated. To ensure saturating dNTP concentrations, 2 mM $T^{H}TP$ and 4 mM $T^{Me}TP$ in case of $RT^{WT}$ and 4 mM $T^{R}TP$ in case of $RT^{M184V}$ were used (see Fig. 5). The *solid* and *dashed lines* show the best fits of the data using a single exponential equation plus slope. *A*, analysis yielded for $RT^{WT}$ rates of 0.07 $s^{-1}$ (± 0.006) for $T^{H}TP$ and 0.03 $s^{-1}$ (± 0.002) for $T^{Me}TP$ incorporation. In the case of $RT^{M184V}$ (*B*) rates of 0.1 $s^{-1}$ (± 0.01) for $T^{H}TP$ and 0.002 $s^{-1}$ (± 0.0002) for $T^{Me}TP$ (*inset*) were obtained.

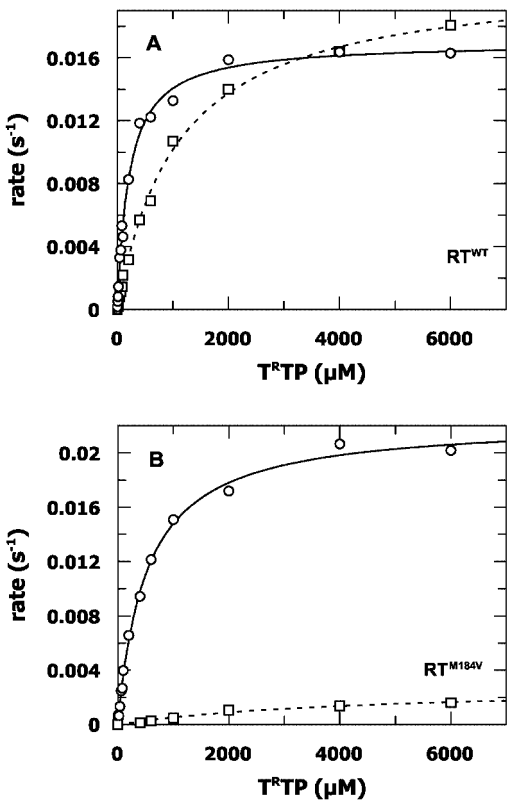


FIG. 5. **Dependence of the pre-steady-state burst rate of misincorporation of $T^{R}TP$ opposite template G on $T^{R}TP$ concentration.** Increasing amounts of $T^{R}TP$ were rapidly mixed with a preformed complex of either 200 nM $RT^{WT}$ (*A*) or $RT^{M184V}$ (*B*) and 100 nM p/t. Reactions were quenched after $t_{1/2}$ of the maximal pre-steady-state rate (see "Experimental Procedures"). Data were fitted to a hyperbolic equation, yielding $K_d$ values for $RT^{WT}$ of 208 $\mu$M (± 14) for $T^{H}TP$ (○) and 512 $\mu$M (± 26) for $T^{Me}TP$ (□), and for $RT^{M184V}$ of 1089 $\mu$M (± 58) for $T^{H}TP$ and > 5000 $\mu$M for $T^{Me}TP$.

ifies that the sugar is also an important element of the substrate recognition process (40).

Analyzing misincorporation of either $T^{H}TP$ or $T^{Me}TP$ opposite template G, the observed effects were even more striking. In this situation the binding affinities as well as the incorporation rates are affected. We interpret this as both nucleotide binding steps (initial loose binding and the induced fit) being involved in substrate selection, thus leading to increased selectivity as compared with correct incorporation. Interestingly, $RT^{M184V}$ only shows a 2.9-fold lower probability to misincorporate $T^{H}TP$ than $RT^{WT}$. This result is in good agreement with findings reported recently (21). However, when it comes to misincorporation of $T^{Me}TP$ the mutant enzyme shows >117-fold higher fidelity. It should be mentioned that this number must be considered as lower limit, since due to substrate inhibition the $K_d$ for "incorrect" $T^{Me}TP$ binding to $RT^{M184V}$ could not be determined accurately (see Table I for details). Thus, we could only determine a lower limit of 5 mM for this $K_d$. As it can be expected from the results shown for $T^{Me}TP$, the $K_d$ values of the two other nucleotides ($T^{Etr}TP$ and $T^{iPr}TP$) are most likely too low to be determined experimentally. Thus, we excluded them from this particular experiment. Taken together, these results show remarkable differences between the two enzymes regarding misincorporation of the steric substrate probe, further supporting, along the lines discussed above, the steric model for DNA polymerase selectivity.

Recently, we presented a detailed steady-state kinetic analysis performing essentially the same kind of experiments as described here (28). As discussed above, the limitation of steady-state kinetic analysis, however, is that only the rate-limiting step of the overall polymerase cycle can be detected. In our case this is dissociation of the enzyme·p/t complex. As long as nucleotide incorporation is faster than dissociation, differences in nucleotide incorporation rates can not be determined applying this approach. In other words, the incorporation rate ($k_{pol}$) is masked by the rate-limiting step of RT·p/t dissociation. For this reason, we did not observe any differences in nucleotide incorporation rates (given as $V_{max}$ in that study; Ref. 28) for the different $T^{R}TP$ substrates performing steady-state measurements. In contrast, as outlined above, performing pre-steady-state kinetic analysis, we observe striking differences for the incorporation rates. Since both sets of experiments, the one described by Strerath *et al.* (28) and the present one, were performed with identical substrates and enzyme batches, they are directly comparable. We therefore believe this is an excellent example showing the benefits of pre-steady-state kinetic measurements to gain insight into complex enzyme mechanisms. Nevertheless, both studies come to the same conclusion, albeit the effects in the steady-state analysis are less apparent compared with the present study.

In conclusion, of the several proposed mechanisms for polymerase fidelity, our data highlight the importance of tight fitting of the nucleotide substrate within the polymerase active site. The presented data provide experimental evidence that minute chances of the overall shape and size of the substrate impose

significant effects on nucleotide selection. This also holds true for alterations of the nucleotide binding pocket around the nascent base pair. Depending on the severity of the structural distortion, both steps of the nucleotide binding pathway, initial binding and induced fit, are involved in discrimination against noncanonical base pairing.

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