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

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Published, JBC Papers in Press, August 27, 2002, DOI 10.1074/jbc.M207854200

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) 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 β-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′-triphosphates (TTP analogues) (26). In these TTP analogues (TRTP) the 4′-hydrogen position of the sugar is substituted with alkyl groups (-CH₃, -CH₂CH₃, and -CH(CH₃)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 T⁵TP (28). Performing steady-state kinetic analyses, we found little difference between both enzymes when promoting "correct" incorporation of the different T⁵TPs. 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.

* This work was supported by European Community Grant QLK2-CT-2001-01451 (to T. R.) and a Deutsche Forschungsgemeinschaft grant (to A. M. J). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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 deoxyribonucleoside triphosphate (dTTP) enters the active site and binds in a two-step process (34). In a first 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 dTTP 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 (26, 36). 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 MgCl₂, and 50 mM KCl. Annealing buffer consisted of 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl.

**Modified Thymidines**—4'-Modified thymidine 5'-triphosphates (dTTPs) were synthesized as described previously (26).

**Oligonucleotides**—Oligodeoxynucleotides were purchased from a commercial supplier and purified by denaturing polyacrylamide gel electrophoresis (15% acrylamide, 7 μm 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'-GTGGCGAATTCGACAGACA and 5'-GTGGTGCTCTGCTGCTGACAGAAATTGTCGACACC (A = A for correct insertion; 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 and 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 appa-

ratus. Products were analyzed by denaturing gel electrophoresis (10% polyacrylamide/7 μm urea) and quantitated by scanning the dried gel using a phosphorimager (Fuji FLA 5000). Data were evaluated using the program Grafit (Ehrithacus Software).

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 dTTP (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 (kₘₐₓ) at the given dNTP concentration were derived from the exponential rates.

Affinities of dTTPs were determined by the dependence of the pre-steady-state burst rate on the dTTP concentrations. To measure the affinities of the dTTPs the preformed p/t-RT (100 and 200 μM complex was rapidly mixed with various concentrations of dTTPs and quenched after t₀ 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 f = ln(1 − ([P]₀/[P]₀))/kₖ₄ ([P]₀)corresponds to the concentration of RT/p/t complex available for incorporation at f = 0 (burst amplitude), and k₆ equals the reaction time (t₀) of the maximal pre-steady-state rate. The observed rates were plotted against the dTTP concentration, and the dissociation constant (Kₐ) 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 dTTP 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 dTTP nucleotides into a 24/36 DNA/DNA p/t substrate by RTWT and RTM184V, 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 dTTP 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.
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Fig. 2. Single turnover, single THTP, or TMeTP incorporation into 24/36 DNA/DNA p/t by HIV-1 RTWT and RTM184V. The curves show the best fit of the data to a double or single exponential equation plus slope. A preformed complex of 200 nm RTWT (A) or RTM184V (B) and 100 nm p/t was rapidly mixed with either 100 μM THTP (○) or 100 μM TMeTP (□). The exponential analysis of the data for RTWT yielded two burst rates (kp01 and kp02) of 0.0002) for THTP and 0.0002) for TMeTP (solid line, double exponential) and a burst rate of 0.5 s⁻¹ (± 0.17) for TMeTP (dashed line, single exponential). The analysis of the experimental data for RTM184V gave burst rates of 0.4 s⁻¹ (± 0.27) and 0.7 s⁻¹ (± 0.1) for THTP (solid line, double exponential) and 0.18 s⁻¹ (± 0.03) for TMeTP (dashed line, single exponential).

than observed previously. This difference can be attributed to different primer length used in these studies.³ Fitting of the experimental data to a double exponential equation plus slope yielded rates (kp01 and kp02) of 0.0002) for THTP and 0.0002) for TMeTP (solid line, double exponential) and a 0.5 s⁻¹ (± 0.17) for TMeTP (dashed line, single exponential).

Upon incorporation of THTP burst rates dropped dramatically for both enzymes (Fig. 2). Fitting of the experimental data to a single exponential equation plus slope yielded rates (kp01) of 0.0002) for THTP and 0.0002) for TMeTP (solid line, double exponential) and a 0.18 s⁻¹ (± 0.03) for RTWT and RTM184V, 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 THTP and TMeTP are listed in Table I.

THTP 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 THTP nucleotide used in this study. Consequently, the rate dependence on concentration for THTP with p/t-bound RT was determined by plotting the observed rates at various concentrations of THTP 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 THTP dissociation constants (Kd values) of 11.7 μM (± 1.4) and 5.5 μM (± 1.0) for RTWT and RTM184V, respectively, consistent with previous measurements (21, 37). Analysis of the binding affinities for TMeTP resulted in Kd values of 19.0 μM (± 1.6) and 16.1 μM (± 3.1) for RTWT and RTM184V, respectively. As anticipated, by further increasing the size of the sugar modification by introducing an ethyl or isopropyl group, the Kd values for these analogues declined gradually with constants of 15.2 μM (± 2.3) and 45.2 μM (± 3.5) for TPrTP and 314.5 μM (± 28.2) and 1001 μM (± 106) for TPrTP (RTWT versus RTM184V).

Single Turnover Nucleotide Misincorporation of THTP 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 THTP opposite template G with both RTs. As described above, 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 THTP and TMeTP by either RTWT or RTM184V. The curves show the best fit to a single exponential equation plus slope. For RTWT we determined incorporation rates of 0.07 s⁻¹ (± 0.0056) and 0.03 s⁻¹ (± 0.0023) for THTP and TMeTP, respectively. The mutant enzyme showed even lower rates of 0.1 s⁻¹ (± 0.01) and 0.002 s⁻¹ (± 0.0002) for THTP and TMeTP. Comparing the relative DNA-dependent DNA replication fidelity of both RTs calculated as ln(Kpol/Kpol)incorrect, the wild-type enzyme shows 12-fold lower misincorporation probability of THTP versus THTP, whereas the mutant enzyme shows a 488-fold lower likelihood. In other words, RTM184V is about 40 times more sensitive toward misincorporation of modified versus unmodified nucleotide.

THTP 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 THTP dissociation constants (Kd) of 208.1 μM (± 14) and 512.6 μM (± 26.6) for RTWT and RTM184V, respectively (Fig. 5). For TMeTP we could derive Kd values of 1089.5 μM (± 58.2) and > 5000 μM for RTWT and RTM184V, respectively. Due to substrate inhibition above 6 mM nucleotide, we can only give a lower limit of 5 μM for the Kd of TMeTP-RTM184V-p/t interaction.

DISCUSSION

In this study we have examined the effect of steric nucleotide probes (THTP) 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 THTP by both enzymes, RTWT and RTM184V, 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-
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 /H9252-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-/H11032-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-/H11032-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 TiPrTP 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 TRTP analogues with significant lower efficiency. There is a 4-fold decrease in RTM184V efficiency compared with RTWT for TMeTP, 13-fold for TEtTP, and 41-fold for TiPrTP (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-

TABLE I

<table>
<thead>
<tr>
<th>TRTP analogue</th>
<th>Kinetic and equilibrium constants for binding and incorporation/misincorporation of TRTP by HIV-1 RT WT and RTM184V</th>
<th>Incorporation efficiency /i.e./</th>
<th>Selectivity factor *</th>
<th>Relative i.e. Selectivity factor</th>
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<td>RTWT</td>
<td>RTM184V</td>
<td>RTWT</td>
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<tr>
<td>THTP</td>
<td>2.99 ± 0.02</td>
<td>1.4</td>
<td>3.1</td>
<td>1.0</td>
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<tr>
<td>TMeTP</td>
<td>5.5</td>
<td>1.0</td>
<td>9.0</td>
<td>0.5</td>
</tr>
<tr>
<td>TEtTP</td>
<td>5.5</td>
<td>1.0</td>
<td>9.0</td>
<td>0.5</td>
</tr>
<tr>
<td>TiPrTP</td>
<td>5.5</td>
<td>1.0</td>
<td>9.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* i.e., incorporation efficiency /kpol/Kd/TTP/*TRTP. Calculated as ([kpol/Kd]TTP/([kpol/Kd]analogue). ** Not measured under saturating nucleotide concentrations due to substrate inhibition.
for TMeTP. The mutant enzyme shows findings reported recently. However, when it comes to not be determined accurately (see Table I for details). Thus, we the two other nucleotides (T EtTP and T iPrTP) are most likely the steric substrate probe, further supporting, along the lines discussed above, the steric model for DNA polymerase selectivity.

Fig. 4. Single turnover kinetics of misincorporation of T>HTP and T>MTP opposite a template G into 24/36 DNA/DNA p/t by HIV-1 RTWT and RTM184V. Preformed complexes of 200 nM enzyme and 100 nM p/t were rapidly mixed with T>HTP (○) or T>MTP (□) and quenched at the time points indicated. To ensure saturating dNTP concentrations, 2 mM T>HTP and 4 mM T>MTP in case of RTWT and 4 mM T>MTP in case of RTM184V were used (see Fig. 5). The solid and dashed lines show the best fits of the data using a single exponential equation plus slope. The analysis yielded for RTWT rates of 0.07 s⁻¹ (± 0.006) for T>HTP and 0.05 s⁻¹ (± 0.002) for T>MTP incorporation. In the case of RTM184V (B) rates of 0.1 s⁻¹ (± 0.01) for T>HTP and 0.002 s⁻¹ (± 0.0002) for T>MTP (inset) were obtained.

Fig. 5. Dependence of the pre-steady-state burst rate of misincorporation of T>HTP opposite template G on T>HTP concentration. Increasing amounts of T>HTP were rapidly mixed with a preformed complex of either 200 nM RTWT (A) or RTM184V (B) and 100 nM p/t. Reactions were quenched after t₁ of the maximal pre-steady-state rate (see “Experimental Procedures”). Data were fitted to a hyperbolic equation, yielding Kᵣ values for RTWT of 208 μM (± 14) for T>HTP (○) and 512 μM (± 26) for T>MTP (□), and for RTM184V of 1089 μM (± 58) for T>HTP and > 5000 μM for T>MTP.

Recently, we presented a detailed steady-state kinetic analysis performing essentially the same kind of experiments as described here. 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 (kinc) is masked by the rate-limiting step of RT:p 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>HTP 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.

Acknowledgments—We thank Roger S. Goody and Michael Famulok for continuous support and Jochen Reinstein and Paul Rothwell for critically reading the manuscript.

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