Kinetic Analysis of Four HIV-1 Reverse Transcriptase Enzymes Mutated in the Primer Grip Region of p66

IMPLICATIONS FOR DNA SYNTHESIS AND DIMERIZATION*

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The highly conserved primer grip region in the p66 subunit of HIV-1 reverse transcriptase (RT) is formed by the β12-β13 hairpin (residues 227–235). It has been proposed to play a role in aligning the 3'-OH end of the primer in a position for nucleophilic attack on an incoming dNTP. To analyze the importance of the primer grip region for nucleophilic attack on an incoming dNTP, to analyze the importance of the primer grip region for nucleophilic attack on an incoming dNTP, to analyze the importance of the primer grip region for nucleophilic attack on an incoming dNTP, to analyze the importance of the primer grip region for nucleophilic attack on an incoming dNTP. To analyze the importance of the primer grip region for nucleophilic attack on an incoming dNTP.

To investigate the importance of the primer grip region for RT function, mutant RTs were used that contain single alanine substitutions of residues Trp229, Met230, Gly231, and Tyr232 in the p66 subunit of the heterodimeric p66/51 enzyme. Steady-state and pre-steady-state kinetic analyses of the enzymes were performed. All mutant enzymes revealed reduced polymerase activity. Mutation of Y232A showed the smallest effect on polymerase function. Equilibrium fluorescence titrations demonstrated that the affinity of the mutants for tRNA was only slightly affected. However, the affinity for primer-template DNA was reduced 27-fold for mutant p66W229A/51 and 23-fold for mutant p66G231A/51, and the maximal pre-steady-state rate of nucleotide incorporation, kcat, was reduced 27-fold for p66W229A/51 and 70-fold for p66G231A/51, respectively. Mutant p66M230A/51 revealed no reduced affinity for primer-template but showed a 71-fold reduced affinity for dTTP. Additionally, the mutations Trp229 and Gly231 affected the stability of the RT heterodimer.

Reverse transcriptase (RT)* is pivotal to the replication of retroviruses. The RT of human immunodeficiency virus 1 (HIV-1) is a heterodimeric enzyme composed of a p66 and a p51 subunit that share a common amino terminus. The smaller subunit is a cleavage product of p66 and lacks the RNase H domain (1, 2). RT is a multifunctional enzyme that possesses a DNA-dependent and an RNA-dependent DNA polymerase activity and an RNase H activity that cleaves RNA in RNA/DNA hybrids. In addition to these activities it uses a host-derived tRNA³* molecule as a primer for the first round of polymerization. Furthermore, it has been shown that HIV RT is only active as a dimeric enzyme and that the monomeric subunits show no catalytic activities (3, 4). For HIV-1 RT and for other DNA polymerases, a polymerization mechanism has been proposed where the binding of primer-template precedes the binding and incorporation of the first dNTP (5). A model for the mechanism of RT polymerization is shown in Scheme 1. In this model, the first step of the reaction is thought to be the formation of a collision complex of RT and DNA that is concentration-dependent. This is succeeded by a conformational change to form a tight complex, which in turn is a prerequisite for dNTP binding. A second conformational change is necessary after the initial binding of the dNTP to form a tight ternary complex. This was identified as the rate-limiting step prior to nucleotide incorporation (6).

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k_{\text{cat}} = \frac{k_1}{k_{-1}} + \frac{K_d}{K_c} \frac{d\text{NTP}}{d\text{NTP}^*}
\]

**Scheme 1. Model for the kinetic mechanism of polymerization by RT.** The asterisks indicate different conformational states of the RT.

One of the highly conserved motifs in retroviral Rtas spans the region from residue 220 to 243 (7). Part of this region in p66 constitutes the "primer grip" and forms a hairpin structure that ranges from residue 227 to 235 (β12-β13 hairpin, adopting the nomenclature of Ref. 8). The corresponding residues in the p51 subunit are unraveled in the crystal structure (9). In the RT/DNA co-crystal the primer grip is located in close vicinity to the polymerase active site and was therefore proposed to be important for maintaining the 3'-OH end of the primer in the appropriate orientation for nucleophilic attack on an incoming dNTP (8, 9). Especially the amino acids in the loop connecting β12 and β13 (Met230 and Gly231) and the residues adjacent to the loop, namely Trp229 at the end of β12 and Tyr232 at the beginning of β13, are close to the nucleic acid and could play a role in orienting the DNA primer. The analysis of regions that are necessary for RT function may contribute to the design of more efficient inhibitors targeting essential residues.

To investigate the importance of the primer grip region for RT function, mutant RTs were used that contain single alanine
substitutions of residues Trp<sup>229</sup>, Met<sup>230</sup>, Gly<sup>231</sup>, and Tyr<sup>232</sup> only in p66. The mutant p66 polypeptides were reconstituted into heterodimer in vitro with a wild type p51 subunit (10–12). Pre-steady-state and steady-state kinetic methods were used to quantify the effects of the mutations on primer-template binding as well as nucleotide binding and incorporation of the mutant enzymes.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Selectively mutated p66/p51 HIV-1 RT and WT RT were prepared by in vitro reconstitution as described previously (10, 11, 13). Briefly, biomass from *Escherichia coli* cultures expressing the mutant p66 RT polypeptide was mixed with that of a strain expressing His<sub>6</sub>-p51 and co-homogenized. The high speed supernatant was loaded on a Ni<sup>2+</sup>-nitritotriacetic acid-Sepharose (Qiagen) column and eluted with a 0–0.5 M imidazole gradient. RT-containing fractions were pooled, dialyzed, and applied on a DEAE-Sepharose column. The flow-through of the column was then applied onto a HiTrap SP column (Pharmacia Biotech Inc.) to separate the dimeric fraction from excess His<sub>6</sub>-p51 and eluted with a 0–0.5 M NaCl gradient. Samples were dialyzed against a buffer containing 50% glycerol and stored at 20 °C.

**Buffers**—RT buffer contained 50 mM Tris-HCl, pH 8.0, 6 mM MgCl<sub>2</sub>, 0.05% (v/v) Triton X-100, 5 mM NaCl, and 5 mM dithiothreitol. Annealing of 20 mM Tris-HCl, pH 7.5, and 50% KCl. Reaction buffer contained 50 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 5 mM KCl, and 1 mM diithioerythritol. HPLC buffer consisted of 50 mM Tris-HCl, pH 8.0, and 150 mM KCl. Urea-loading buffer contained 7 M urea in Tris borate/EDTA buffer with 0.1% xylene cyanol and bromphenol blue.

**Polymerase Activity Determination**—RT polymerase activity on poly(rA)/oligo(dT)<sub>12–18</sub> substrates was measured by a standard assay (30 µl reaction volume) described previously (11) with 5–10 ng of enzyme in RT buffer. Under these conditions, 1 unit of RT activity catalyzes the incorporation of 1 nmo1 of dTTP into poly(rA)/oligo(dT)<sub>12–18</sub> in 10 min.

**HPLC Gel Filtration Analysis**—HPLC gel filtration analysis was performed using a Superdex 200 HR10/30 column. 5–50 µg of enzyme were loaded onto the column. The column was eluted with HPLC buffer at 0.5 ml/min.

**tRNAs**—In vitro transcription and purification of tRNAs<sub>34</sub> was performed as described previously (14). *E. coli* tRNAs<sub>34</sub> was purchased from Sigma.

**Fluorescence Equilibrium Titrations with tRNA**—Fluorescence titrations were performed using an SLM Smart 8100 spectrofluorometer equipped with a PH-PC 9635 photomultiplier. Intrinsic fluorescence measurements using the tryptophan fluorescence of the enzymes were performed in reaction buffer in a total volume of 0.7 ml or 1 ml as described previously at an excitation wavelength of 295 nm. The emission intensity was measured at 340 nm (slit widths set at 1 and 8 nm for excitation and emission, respectively). For displacement titrations, a preformed complex of 30 nM RT and 30 nM of fluorescently labeled p/t DNA was titrated with unlabeled p/t in reaction buffer. The increase in fluorescence was used as a signal for displacement of labeled p/t. Data were analyzed by using the fitting program Scientist to determine the K<sub>d</sub> values for the unlabeled p/t.

**5’-End Labeling of Primers**—Primer oligodeoxynucleotides were 5’-end-labeled with T4 polynucleotide kinase (New England Biolabs) using 7 µCi of [γ-<sup>32</sup>P]ATP (3000 Ci/mmol) (Hartmann Analytic, Braunschweig) per 100 pmol of DNA for 1 h at 37 °C. The primer was separated from nucleotides by purification over a NucTrap Probe Purification Column (Stratagene) according to the procedure described by the manufacturer. Concentrations were determined by thin layer chromatography using a phosphor imaging device (Bio-Rad) for quantification.

**Rapid Kinetics of Nucleotide Incorporation**—Rapid quench experiments were carried out in a rapid quench apparatus built by KinTek Instruments (University Park, PA) in reaction buffer. 15 µl of a preformed complex of 100 nM RT or higher concentrations as indicated for the mutants in the figures and p/t DNA (200 nM) were rapidly mixed with 15 µl of dTTP (100 or 450 nM) for the various enzymes. Reactions were quenched at various time points with 0.6% trifluoroacetic acid. The products were analyzed on denaturing 10% polyacrylamide gels with 7 x urea. Quantification of the data was achieved by phosphor imaging. All concentrations reported are final concentrations after mixing in the rapid quench apparatus. Data were fitted to a burst equation (single exponential plus slope) using the program Grafit. The pre-steady-state rate is given by the exponential (6). The burst amplitude corresponds to the amount of RT bound to p/t at the beginning of the reaction (t = 0).

**Affinities for dTTP** were determined by the dependence of the pre-steady-state burst rate on the dTTP concentration. The RT-p/t complex (200 nM p/t with RT as indicated in Fig. 5 for the various enzymes) was rapidly mixed with increasing concentrations of nucleotide and quenched after t<sub>1/2</sub> of the maximal pre-steady-state rate. The corresponding rates were calculated from the elongated fraction of the primer by converting the single exponential equation into k = ln1(1) (fraction of elongated primer/burst amplitude)/t<sub>1/2</sub>. The observed rates were then plotted against the nucleotide concentration, and the K<sub>d</sub> for dTTP was calculated by fitting the data to a hyperbola using the program Grafit.

**Primer Extension Assays**—Primer extension assays were performed with 300 nM radioactively labeled p/t and 100 or 450 nM dimeric RT enzyme at 25 °C in reaction buffer in a total reaction volume of 10 µl. Reactions were started by adding dNTPs as indicated in Fig. 6 and were stopped after 5 min by the addition of 10 µl of urea-loading buffer.

**RESULTS**

**Effect of Mutations on RT Polymerase Activity**—To obtain a first impression of whether the introduction of mutations into the primer grip region has an impact on RT function, we assayed the RNA-dependent DNA-polymerase activity of the reconstituted wild type and mutant enzymes on the homopolymeric substrate poly(rA)/oligo(dT)<sub>12–18</sub>. Table 1 shows that the RT activity of all mutants is reduced. The most severe reduction of RT polymerase activity can be detected with p66<sup>W229A/Y232A</sup>. Previous primer extension experiments with a synthetic 36/71-mer p/t DNA had already indicated that this mutant is strongly impaired in polymerase activity (11). However, no detailed kinetic analyses of the polymerase function of the mutants were available.

| Enzyme   | Percentage of Wild type activity |
|----------|----------------------------------|
| WT p66/p51 | 100                              |
| p66<sup>W229A</sup>/p51 | 0.1                              |
| p66<sup>Y232A</sup>/p51 | 11                               |
| p66<sup>W229A/Y232A</sup>/p51 | 21                               |
| p66<sup>Y232A</sup>/p51 | 34                               |

This table summarizes the effects of mutations on the polymerase activity of HIV-1 RT. The decrease in activity is expressed as a percentage of the wild type activity.
the dimer stability of p66/51. Second, the binding affinity to the nucleic acid substrate can be reduced; and third, the mutant enzyme can have a defect in nucleotide binding and incorporation (see Scheme 1). In the following sections we describe the analysis of these possibilities.

**Effect on Dimer Stability**—Since monomeric HIV-1 RT subunits do not show catalytic activity (3), it is important to determine the dimer content of the purified enzymes, especially since the enzymes used were reconstituted from subunits expressed separately.

N-terminal tagging of the p51 subunit with six additional histidines allows purification by metal chelate chromatography. The wild type or mutated p66 subunit, which in our case does not have a histidine extension, can only be retained on the column if it interacts with p51 (10). Although metal chelate chromatography (16) of the *in vitro* reconstituted HIV-1 RT mutants is a powerful method that permits purification of the dimer fraction of an enzyme, it does not give much information on the stability of the purified dimer. If the dimer is not stable, a dimer/monomer equilibrium will be reestablished after purification of the dimeric enzyme fraction. To investigate this possibility, we determined the dimeric content of the RT enzymes by HPLC size exclusion gel filtration (Fig. 1) after the proteins had been purified and dialyzed against storage buffer. The results, shown in Table II, indicate that the mutants p66W229A/51 and p66G231A/51 are not completely dimerized. p66W229A/51 is only about 35% dimeric, and the dimer content of p66G231A/51 is about 60%. Therefore, the low polymerase activity of mutants p66W229A/51 and p66G231A/51 is partly due to the low dimer content of the purified stock solutions. This result indicates that the dimer, due to its instability, tends to monomerize during the purification procedure. To account for this result, for all the analyses shown below only the dimer content of the enzymes was considered for calculations. The dimer interface of the mutants p66M230A/51 and p66Y232A/51 appears to be intact, since we could not detect appreciable amounts of monomer.

To examine whether the enzymes p66W229A/51 and p66G231A/51 are stable under the conditions used in our kinetic experiments (25 °C for up to 1.0 h) we isolated the dimer fraction of mutants p66W229A/51 and p66G231A/51 by separation on an HPLC gel filtration column. This is shown in Fig. 1, C and D, for p66W229A/51. Immediately after collection, an aliquot of the collected dimer fraction was loaded again on the HPLC gel filtration column to show the dimer content of the isolated peak fraction (Fig. 1C). Another aliquot was incubated for 1.0 h at 25 °C and then analyzed again by HPLC gel filtration (Fig. 1D). The mutant enzymes remained dimeric for up to 4.5 h of incubation at 25 °C (data not shown). This suggests that the proteins do not dissociate to a great extent under the conditions in which our experiments were performed. However, the wild type and mutant enzymes started to dissociate appreciably after 15 min at 37 °C, a temperature that in general is used for many enzyme assays with RT (data not shown).

**tRNA Binding Is Not Impaired**—tRNA<sub>Lys</sub> is the natural replication primer of HIV-RT. We have shown recently that the intrinsic fluorescence of RT can be used to monitor p/t and tRNA binding (14, 17). Heterodimeric RT possesses 37 tryptophan residues. Binding of tRNA or p/t to RT leads to a quenching of the tryptophan fluorescence of the protein that can be used to determine the dissociation constant ($K_d$). However, comparison of binding affinities for WT RT to various tRNA species showed that RT does not bind tRNA<sub>Lys</sub> specifically; instead, for all tRNA species tested, similar $K_d$ values were determined (14). Therefore, to determine whether the mutant RTs are still able to bind tRNA we used *E. coli* tRNA<sub>Tyr</sub> for the

![Fig. 1. HPLC gel filtration of purified RTs.](image)

The dimer content of the enzymes was measured by HPLC size exclusion chromatography at room temperature. The retention time for the heterodimeric p66M230A/51 was 25.3 min (A), and the retention times for p66W229A/51 (B) were 25.15 min for the heterodimer, 27.13 min for p66, and 28.77 min for p51. To analyze the stability of the dimeric fraction, the dimer peak of p66W229A/51 was isolated and applied to the column directly after collection (C) or 1 h after collection and incubation at 25 °C (D).
intrinsic fluorescence titrations (Fig. 2). The results presented in Table III indicate that mutations in the primer grip do not affect tRNA binding. This was confirmed by titration of wild type and mutant RT p66<sup>W229A/51</sup> with synthetic tRNA<sub>Lys</sub>. The K<sub>d</sub> values obtained for synthetic tRNA<sub>Lys</sub> were 11 nM for the wild type and 7 nM for p66<sup>W229A/51</sup>. Our results demonstrate that the primer grip region is not essential for tRNA binding.

**Influence on Primer-Template Binding**—The affinity of the mutant RTs for p/t DNA was determined by equilibrium fluorescence titrations using a 18/36-mer p/t DNA substrate labeled at the 5′-end of the primer with the fluorescein derivative FAM. The K<sub>d</sub> values obtained for the wild type enzyme with this method are similar to those obtained by intrinsic fluorescence measurements (K<sub>d</sub> = 1.8 nM; Ref. 17). Since some of the mutants have rather high K<sub>d</sub> values for p/t, for practical reasons we used the extrinsic fluorescence determination for standard procedures because the signal change is larger and more stable (Fig. 3). The binding affinities for p66<sup>W229A/51</sup> and p66<sup>G231A/51</sup> were about 23–27-fold lower than for wild type, whereas the binding affinity for mutant p66<sup>Y232A/51</sup>, which possessed the highest RT polymerase activity (Table I), was only about 8-fold lower. Interestingly, the mutation of p66<sup>M230A/51</sup> does not appear to have an effect on p/t binding. This result indicates that this mutant, which retains only 11% of the WT RT polymerase activity, must be impaired in one of the steps following p/t binding. However, to exclude the possibility that the fluorescence label has an influence on the affinity of the mutant for p/t, we performed displacement titrations. A preformed complex between RT and the fluorescent p/t was titrated with the unlabeled 18/36-mer DNA. Displacement of the labeled p/t is dependent on the K<sub>d</sub> of both p/t DNAs. Analysis of the displacement data for the wild type and p66<sup>M230A/51</sup> leads to K<sub>d</sub> values for the unlabeled p/t species of 5.2 and 5.3 nM, respectively, which indicates that the label does not influence the binding affinities of the mutant and confirms that the affinity of wild type and p66<sup>M230A/51</sup> for p/t is unchanged.

![Image](image_url)
The observed rates at different dTTP concentrations were plotted against the dTTP concentration, and the dissociation constant ($K_d$) for dTTP was calculated by fitting the data to a hyperbola (6) (Fig. 5). Again, mutant p66Y232A/51 was the least impaired. In addition to their low affinity for p/t DNA, the affinity of mutants p66W229A/51 and p66G231A/51 for dTTP was reduced 20- and 36-fold, respectively. Our data clearly show that the major effect of the mutation in p66M230A/51 is a severe increase (71-fold) in the $K_d$ for dTTP. A summary of the results is given in Table IV.

**DISCUSSION**

The aim of this work was to characterize the importance of the primer grip of HIV-1 RT by kinetic analyses of four mutant RT enzymes. Comparison of the primer grip region of HIV-1 RT with RTs from other retroviruses possessing a p66/51 structure (feline immunodeficiency virus, simian immunodeficiency virus, equine infectious anemia virus, and HIV-2) shows that

![FIG. 4. Comparison of the kinetics of dTTP incorporation at 1 mM dTTP. A, a preformed complex of 200 nM p/t DNA and 100 nM WT RT (●, p66p230A/51) or p66Y232A/51 (○) was mixed with dTTP. B, a preformed complex of 200 nM of p/t DNA and 300 nM of p66W229A/51 (○) or 200 nM of p66G231A/51 (▲) was mixed with dTTP. Data were fitted to a single exponential plus slope. The maximal burst rates obtained at saturating dTTP concentrations ($k_{pol}$) are shown in Table IV.](image-url)

**TABLE IV**

| Enzyme       | $k_{pol}$ | $K_d$ | $k_{pol}/K_d$ |
|--------------|-----------|-------|---------------|
| wt p66/51    | 26        | 9     | 2.88          |
| p66W229A/51  | 0.96      | 184   | 5.22 x 10^{-3}|
| p66M230A/51  | 5.1       | 640   | 7.97 x 10^{-3}|
| p66G231A/51  | 0.37      | 325   | 1.4 x 10^{-3} |
| p66Y232A/51  | 4.7       | 15    | 0.31          |

* Corrected for dimer content.

![FIG. 5. Dependence of the pre-steady-state burst rate on dTTP concentration. Increasing amounts of dTTP rapidly mixed to a pre-incubated solution of 200 nM p/t and 100 nM of p66Y232A/51 (○) or p66M230A/51 (■) or 300 nM of p66W229A/51 (○). The reactions were stopped at a defined time point, and data were fitted to a hyperbola (see “Experimental Procedures”), which yielded $K_d$ values for dTTP as shown in Table IV for all enzymes.](image-url)
fails to yield heterodimeric RT (12). Crystallographic data from unliganded HIV-1 RT show that the side chains of Trp \(^{229}\), Met \(^{230}\), and Tyr \(^{232}\) are oriented into the core region between the primer grip and the polymerase active site (22). We therefore assume that the mutations in Trp \(^{229}\), Gly \(^{231}\), or Leu \(^{234}\) indirectly influence dimerization, for example by changing or preventing interactions with amino acids that contact the dimer interface. Fig. 7 shows possible interactions of the amino acids of the primer grip with surrounding residues as determined by the RT crystal structure of Rodgers et al. (22). A contact of Trp \(^{229}\) with Pro \(^{95}\) (distance \(-3.79\) Å) in the palm subdomain of p66 is possible by van der Waals interactions. Pro \(^{95}\) is also involved in binding NNIs (19, 20, 23). Pro \(^{95}\) in turn may interact with Asn \(^{137}\) and Glu \(^{138}\) in the finger subdomain of p51 (distance \(-3.81\) Å). By changing the bulky Trp \(^{229}\) into Ala or by exchanging Leu \(^{234}\) to Ala, a “deficiency of atoms” occurs in the pocket harboring these amino acids. This could lead to a conformational change of adjacent residues to fill the created space. As a consequence, the loop (residues 135–140) between β7 and β8 (shown in light purple in Fig. 7) of p51 containing Asn \(^{137}\) and Glu \(^{138}\) (highlighted in yellow in Fig. 7) and reaching into p66 is not able to contact the corresponding residues in p66 properly. Furthermore, this effect could lead to displacement of the β7-β8 loop of p51 away from the pocket, thus destabilizing the dimer interface. At present we are investigating the role of this loop region of p51 in dimer formation. Interestingly, the only resistance mutation against NNIs found in p51 is located in this region (residue Glu \(^{138}\) → Lys), indicating a function of this loop in binding the NNI (24, 25).

For residue Gly \(^{231}\), the situation might be somewhat different. It is located in the loop connecting β12 and β13 and therefore might have an important architectural role for the stability of the β12-β13 hairpin. Exchanging this residue against the bulkier alanine could affect the stability of the hairpin, which in turn by a change in conformation of surrounding residues could have similar consequences for dimer stability as already described above.

Additionally, our results demonstrate that the conformational changes described influence the polymerization mechanism. Our experiments indicate a function for Trp \(^{229}\) and Gly \(^{231}\) in p/t binding and dNTP incorporation. Crystallographic data have implicated the primer grip in orienting the primer terminus for nucleophilic attack on an incoming dNTP (8). Our results with the mutants p66 \(^{W229A/51}\) and p66 \(^{G231A/51}\) imply that these enzymes are unable to align the primer properly, leading to a reduced affinity for p/t and dNTP and to a reduced incorporation rate for dNTP. However, the mutation in Met \(^{230}\) clearly shows that the two effects, i.e. p/t binding and dNTP incorporation, can be separated. With mutant p66 \(^{M230A/51}\), dNTP binding and incorporation are impaired, but we do not find a significant reduction of the affinity for p/t DNA (Table III). Modeling experiments that dock a dTTP into the polymerase active site of the HIV-1 RT/DNA/Fab cocrystal suggest that β-strand β6 (residues 105–118), the β9-β10 hairpin (residues 178–191), and the region β11a-β11b (residues 214–222) of p66 might be involved in dNTP binding (9, 26). This allows positioning of the β- and γ-phosphate of the dTTP to the active site residues Asp \(^{185}\) and Asp \(^{186}\). Direct interaction of the primer grip hairpin with a dNTP would be unfavorable, since the base of the dTTP in this case could not stack onto the
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terminal base of the primer. Therefore, it is reasonable to assume that the amino acids of the primer grip are not directly involved in dNTP binding. Instead, the changes in the orientation of the primer induced by the mutations probably lead to a less favorable positioning of the α-phosphate of the dNTP for attack by the 3′-OH of the primer terminus. However, minor changes of the primer orientation do not necessarily lead to a reduced affinity of the mutated enzyme for p/t but can have an affect on the stacking of the incoming nucleotide onto the terminal base of the primer. This is probably the case with mutant p66G231A/51. This effect could also lead to a reduced fidelity of the mutant enzymes. In fact, preliminary results with mutant p66W229A/51 indicate that the fidelity of this enzyme has also changed (data not shown).

Comparison of the results obtained for tRNA binding with those of p/t binding demonstrate that in the binary complex the primer grip region is not important for the affinity of RT for tRNA. We have shown previously by displacement titrations that a p/t DNA can be displaced by tRNA_Lys and various other tRNAs (14). This implies that tRNA and p/t bind to the same or at least partially overlapping sites. Together with the data presented here, these experiments suggest that the binding sites for tRNA and p/t are overlapping but not identical. A function of the primer grip in tRNA binding must be expected when the tRNA is hybridized to the primer binding site on the viral RNA, since here its 3′-OH end is used as a primer for the start of minus strand synthesis.

Previous results indicate that introduction of the mutation G231A into the virus leads to infectious particles in cell culture assays, whereas after introduction of W229A infectious virus is not formed (11, 12). Our results show that in vitro both mutants are significantly impaired in polymerase function. The reason for this discrepancy is not clear, since most parameters we have measured for these two mutants (Tables I–IV) are similar. The only major difference (Table I) is in the steady-state activity assay, in which p66W229A/51 is considerably worse than p66G231A/51. This is initially surprising, since the single nucleotide incorporation kinetics of p66W229A/51 are better than those of p66G231A/51. We attribute this discrepancy to the poorer dimerization properties of p66W229A/51, which probably lead to extensive dissociation under the low concentration conditions of the steady-state assay. In contrast, the quenched flow results were obtained at much higher protein concentration and are corrected for dimer content. We therefore conclude tentatively that impairment of heterodimer formation may be responsible for the lack of production of infectious virus for this mutant. Another possibility that has not been analyzed yet are compensatory mutations that could have occurred during the cell culture experiment to overcome the impairments of p66G231A/51. In the absence of this, production of infectious virus after introduction of the G231A mutation remains a surprising result in view of the significantly impaired kinetic properties.

The observation that the mutation Y232A leads to a decrease in infectious particles in cell culture assays, although in in vitro assays the polymerization activity is not as drastically reduced as with mutant p66G231A/51, is probably due to the impaired RNase H activity of p66Y232A/51 demonstrated previously (12). One general result of the work presented here is that single amino acid changes in HIV-1 RT can have far reaching and unexpected global effects on enzyme structure and function (e.g. dimerization), which can make interpretation of results difficult and misleading unless these factors are specifically taken into account. Such unexpected results can, however, be useful. As an example, the information obtained on the effects of mutations of the primer grip region on dimerization suggest that this region of the molecule might be included in the search for approaches to inhibition of dimerization.

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REFERENCES

1. Di Marzo Veronese, F., Copeland, T. D., DeVico, A. L., Rahman, R., Oroszlán, S., Gallo, R. C., and Sarngadharan, M. G. (1986) Science 231, 1289–1291
2. Lightfoot, M., Coligan, J., Folks, T., Feizi, A. S., Martin, M. A., and Venkatases, S. (1986) J. Virol. 60, 771–775
3. Restle, T., Müller, B., and Goody, R. S. (1990) J. Biol. Chem. 265, 8886–8898
4. Restle, T., Müller, B., and Goody, R. S. (1992) FEBS Lett. 300, 97–100
5. Rittinger, K., Devita, J., and Goody, R. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8046–8049
6. Kati, W. M., Johnson, K. A., Jervsa, L. F., and Anderson, K. S. (1992) J. Biol. Chem. 267, 25988–25997
7. Xiong, Y., and Eckehak, T. H. (1990) EMBO J. 9, 3353–3362
8. Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hirt, A., Hughes, S. H., and Arnold, R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6330–6334
9. Nanni, R. G., Ding, J., Jacobo-Molina, A., Hughes, S. H., and Arnold, E. (1993) Antimicrob. Agents Chemother. 37, 3098–3105
10. Jacques, P. S., Wohrl, B. M., Howard, K. J., and Le Grice, S. F. (1994) J. Biol. Chem. 269, 1388–1393
11. Jacques, P. S., Wohrl, B. M., Ottmann, M., Darlix, J.-L., and Le Grice, S. F. (1994) J. Biol. Chem. 269, 26472–26479
12. Ghosh, M., Jacques, P. S., Rodgers, D. W., Ottman, M., Darlix, J. L., and Le Grice, S. F. (1996) Biochemistry 35, 8553–8562
13. Le Grice, S. F. J., Naas, T., Wohlgensinger, B., and Schatz, O. (1991) EMBO J. 10, 3905–3911
14. Thrall, S. H., Reinstein, J., Wohrl, B. M., and Goody, R. S. (1996) Biochemistry 35, 4609–4618
15. Müller, B., Restle, T., Reinstein, J., and Goody, R. S. (1991) Biochemistry 30, 3709–3715
16. Hochuli, E., Bannwarth, W., Dobeli, H., Genta, R., and Stuber, D. (1988) Bio/Technology 6, 1321–1325
17. Devita, G., Müller, B., Immendorfer, U., Gautel, M., Rittinger, K., Restle, T., and Goody, R. S. (1993) Biochemistry 32, 7966–7971
18. Smerdon, S. J., Jager, J., Wang, J., Kohlstaedt, L. A., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3911–3915
19. Ding, J., Das, K., Moereels, H., Koymans, L., Andries, K., Janssen, P. A., Hughes, S. H., and Arnold, E. (1995) Nat. Struct. Biol. 2, 407–415
20. Ren, J., Esnouf, R., Garman, E., Somers, D., Ross, C., Kirby, I., Keeling, J., Darby, G., Jones, Y., Stuart, D., and Stammers, D. (1995) Nat. Struct. Biol. 2, 293–302
21. Erickson, J. W., and Burt, S. K. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 545–571
22. Rodgers, D. W., Gamblin, S. J., Harris, B. A., Ray, S., Culp, J. S., Hellmig, B., Woolf, D. J., Debecik, C., and Harrison, S. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1222–1226
23. Ren, J., Esnouf, R., Hopkins, A., Ross, C., Jones, Y., Stammers, D., and Stuart, D. (1995) Structure 3, 915–926
24. Boyer, P. L., Ding, J., Arnold, E., and Hughes, S. H. (1994) Antimicrob. Agents Chemother. 38, 1909–1914
25. Jonckheere, H., Taymans, J.-M., Balzarini, J., Vela´zquez, S., Camarasa, M.-J., Venkatesh, H., Debouck, C., and Harrison, S. C. (1994) J. Biol. Chem. 269, 25255–25258
26. Patel, P. H., Jacobo-Molina, A., Ding, J., Tantillo, C., Clark, A. D. Jr., Raag, R., Nanni, R. G., Hughes, S. H., and Arnold, E. (1995) Biochemistry 34, 5351–5363