Role of the “Helix Clamp” in HIV-1 Reverse Transcriptase Catalytic Cycling as Revealed by Alanine-scanning Mutagenesis*

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Residues 259–284 of HIV-1 reverse transcriptase exhibit sequence homology with other nucleic acid polymerases and have been termed the “helix clamp” (1). Alanine-scanning mutagenesis has previously demonstrated that several residues in a-H make important interactions with nucleic acid and influence frameshift fidelity. To define the role of a1 (residues 278–286) during catalytic cycling, we performed systematic site-directed mutagenesis from position 277 through position 287 by changing each residue, one by one, to alanine. Each mutant protein was expressed and, except for L283A and T286A, was soluble. The soluble mutant enzymes were purified and characterized. In contrast to alanine mutants of a-H, alanine substitution in a1 did not have a significant effect on template primer (T-P) binding as revealed by a lack of an effect on Kₐ(T-P), Kₐ, for 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate, K₀(T-P), and processivity. Consistent with these observations, the fidelity of the mutant enzymes was not influenced. However, alanine mutagenesis of a1 lowered the apparent activity of every mutant relative to wild-type enzyme. Titration of two mutants exhibiting the lowest activity with T-P (L282A and R284A) demonstrated that these mutant enzymes could bind T-P stoichiometrically and tightly. In contrast, active site concentrations determined from “burst” experiments suggest that the lower activity is due to a smaller population of enzyme bound productively to T-P. The putative electrostatic interactions between the basic side chains of the helix clamp and the DNA backbone are either very weak or kinetically silent. In contrast, interactions between several residues of a-H and the DNA minor groove, 3′-5 nucleotides from the 3′-primer terminus, are suggested to be critical for DNA binding and fidelity.

The type 1 human immunodeficiency (HIV-1) reverse transcriptase (RT) is a DNA polymerase that utilizes both RNA and DNA templates to accomplish genomic replication. Crystallographic structures of RT complexed with a non-nucleoside inhibitor (1, 2) or DNA (3) have been solved. Reverse transcriptase is a heterodimer of 66- and 51-kDa polypeptides. The p51 subunit is a carboxyl-terminal truncation of the p66 subunit and although the amino-terminal sequence of p66 is identical to p51, the tertiary organization of the two subunits differ (4). The polymerase domain of the p66 subunit forms a nucleic acid binding cleft, and by analogy with a right hand, its three subdomains are referred to as fingers, palm, and thumb (4). Each subunit of the heterodimer also has a “connection” subdomain that forms the “floor” of the nucleic acid binding site (5). The carboxyl terminus of the p66 subunit has a fifth subdomain with RNase H activity that cleaves the RNA strand of hybrid duplex. As the nucleic acid binding cleft in the p51 polymerase domain is occluded by the connection subdomain, it lacks a functional “active site” in the heterodimer.

Crystallographic data suggest that two antiparallel a-helices in the thumb subdomain interact 3–5 (a-H) and 6–9 (a1) nucleotides from the polymerase active site with the primer and template strands, respectively (Fig. 1). Within this region, the DNA is observed to be bent 40–45° and undergoes a transition from A- to B-form (3). The primary sequence in the vicinity of these a-helices has been found to have sequence homology with several other nucleic acid polymerases and has been termed the “helix clamp” (6). Alanine-scanning mutagenesis has demonstrated that a-H plays an important role in template primer (T-P) binding and fidelity (7, 8). The periodicity of the effects observed suggested that a short segment of one face of a-H (i.e. Gln258, Gly262, Trp266) interacts with the T-P. Kinetic parameters for T-P observed with the alanine mutants of Gly262 and Trp266 indicate that interactions in the minor groove of the duplex, several nucleotides from the primer-terminus, influence processivity and fidelity through T-P slippage (8). These results support earlier observations that processive synthesis by RT is dependent on the DNA sequence of the first 6 base pairs of the duplex primer stem and that single base pair differences in this region can affect both processivity (9) and frameshift fidelity (10).

The DNA in the crystal structure of the RT-DNA-Fab complex was too short to determine whether nucleic acid interactions occur in other subdomains of p51 (3). However, modeling longer stretches of nucleic acid into the binding cleft has suggested that residues of both a-H and a1 of the p51 thumb subdomain may also interact with nucleic acid (5, 11). Recent modeling of a RNA templateprimer into the nucleic acid binding cleft has suggested that interactions may occur between the RNA primer strand and a1 (6). The primary sequence in the stretch of residues comprising a-H and a1 is highly basic (Lys259, Lys262, Lys275, Arg277, Lys281, Arg284, Lys287), and most of these residues are observed to be facing the DNA

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§The abbreviations used are: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; RNase H, ribonuclease H; p66, 66-kDa RT polypeptide; p51, 51-kDa carboxyl-terminally processed p66; AZTTP, 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; T-P, template primer; Cα, α-carbon.
phosphate backbone (Fig. 1). To define the role of \( \alpha \) during catalytic cycling, we performed systematic site-directed mutagenesis from position 277 through 287 by changing each residue, one by one, to alanine. Each mutant enzyme was expressed and purified to near-homogeneity, and their specific activities, substrate kinetic parameters, and fidelity surveyed.

**EXPERIMENTAL PROCEDURES**

Materials—T4 polynucleotide kinase, poly(rA), (dT)\(_{20}\), and dNTPs were from Pharmacia Biotech Inc. \([\alpha-\text{32P}]dTT\) (3000 Ci/mmol) was from DuPont NEN. Sodium heparin (170 USP units/mg) was from U. S. Biochemical Corp. High performance liquid chromatography-purified synthetic oligonucleotides were purchased from Genosys (Woodlands, TX).

Mutagenesis of the RT Gene—To change specific residues in the HIV-1 coding sequence, oligonucleotide-directed mutagenesis was performed as described by Kunkel et al. (12). The following mutations were introduced into M13-RT vector as described by Goel et al. (13), 5’ to 3’: R277A (AGG to GCG), Q278A (CAA to GCA), L281A (AAA to GCA), R284A (AGA to GCA), and R277A (AGG to GCG). Q278A (CAA to GCA), L281A (AAA to GCA), and K287A (AAA to GCA). The complete coding sequence of the RT gene was sequenced to confirm the presence of the desired substitution and the absence of any other change.

Protein Purification—Alanine mutants were purified as described previously (7), and the purified mutant enzymes were assayed for exonuclease activity. Additional purification to separate p66 homodimer, p66/p51 heterodimer, and/or exonuclease contaminants was accomplished with a single-stranded DNA-cellulose column as described (7). The amount of contaminating 3’ → 5’ exonuclease activity on a mismatched primer for each enzyme preparation was determined as described (14). Enzyme preparations had at least 10-fold lower exonuclease activity than Klenow fragment.

Reverse Transcriptase Polymerization Assays—Enzyme activities, substrate kinetic parameters, and fidelity surveyed.

**FIG. 1. Interaction between template-primer and m.H.I.** The position of the phosphates of the DNA and the Cys of \( \alpha \) and \( \alpha I \) are from the coordinates obtained from the Protein Data Bank (PDB file 1H1M, see Ref. 3). The DNA phosphates and Cys of the basic residues are highlighted as spheres, and the 3’-end of the primer strand is indicated. The bonds closest to the viewer are thickest.

Enzyme concentrations were determined from protein determinations (16) which had been calibrated by amino acid analysis.

The dissociation rate constant \( (k_{\text{d}}) \) for poly(rA)-oligo(dT)\(_{20}\) was determined as described (15). Briefly, enzyme was preincubated with T-P for 5 min before challenging free polymerase with heparin (zero time). At time intervals after adding challenge, 20-\( \mu l \) aliquots were removed and mixed with 20 \( \mu l \) of dTTP/Mg\(^{2+}\) to determine the concentration of RT remaining bound to T-P. After an additional 10-min incubation, the reaction was stopped with EDTA. The final reaction conditions were 150 nm RT, 150 nm T-P (expressed as primer 3’ termini), 50 nm Tris-HCl, pH 7.4, 10 nm MgCl\(_2\), 30 \( \mu l \) [\( \alpha-\text{32P}]dTT\) and 1 mg/ml heparin.

Titration of enzyme with T-P (i.e. poly(rA)-oligo(dT)\(_{20}\)) was performed as before (15). To determine the concentration of RT bound to T-P, a 40-\( \mu l \) solution of 32 nm enzyme, expressed as concentration of dimer, was preincubated with varying concentrations of poly(rA)-oligo(dT)\(_{20}\). After 10 min, the reaction was initiated with 10 \( \mu l \) of 5 m g/ml heparin and 150 \( \mu l \) [\( \alpha-\text{32P}]dTT. After an additional 10 min, the reaction was stopped by the addition of 20 \( \mu l \) of 0.5 mM EDTA and the amount of incorporation was determined as above. Under these conditions, total incorporation is proportional to the concentration of RT-T-P complex.

To determine the active site concentration of enzyme, synthesis was limited to a single deoxynucleotide by using a 47/24-mer heteropolymeric template primer and including only the complementary dTTP to the first template base. The sequence of the primer and template were 5’-GCTTGCATGGGTTACGTGCTTG-3’ and 3’-CGACCTACGGA-GTGTCACCAGAACGTACGGA-5’. When product release is the rate-limiting step during catalytic cycling, then a burst of product formation is observed upon initiation of the reaction (Scheme I, \( k_4 \ll k_3 \)).

The amount of product formed during the burst should be equivalent to the concentration of active enzyme and has been observed previously (17–20). Reactions were initiated by the addition of enzyme and 30-\( \mu l \) aliquots withdrawn at time intervals, stopped with EDTA, and incorporation determined by filter binding as described above. The final reaction conditions were 560 nm dimeric RT, 2 \( \mu l \) T-P (expressed as primer 3’ termini), 50 nm Tris-HCl, pH 7.4, 150 mM KCl, 5 mM MgCl\(_2\), 10 \( \mu l \) [\( \alpha-\text{32P}]dTT.

Fidelity Assays—The in vitro fidelity of DNA-dependent DNA synthesis was measured using a M13mp2 DNA substrate containing a 390-nucleotide single strand gap spanning the lacZ\( \alpha \) complementation gene target as described previously (21). DNA synthesis reactions (25 \( \mu l \) contained 35 fmol of gapped M13mp2 DNA, 1–5 pmol of wild-type or mutant RT, 1 \( \mu l \) each of the four dNTPs in 20 nm Tris-HCl, pH 8.0, 10 nm MgCl\(_2\), and 2 m dithiothreitol. Reactions were incubated at 37°C for 60 min and terminated by addition of EDTA. Complete gap-filling synthesis was confirmed by electrophoretic analysis of the products on a 0.8% agarose gel.

Processivity Analysis—Termination probability was analyzed using a primed single-stranded heteropolymeric M13mp2 DNA substrate as described previously (9). The [\( \alpha-\text{32P}]ATP 5’-end-labeled primer used for this analysis was complementary to lacZ positions 105–120. Reactions conditions were the same as for the DNA synthesis reaction, except the DNA (160 fmol) was present in 3-fold molar excess over wild-type enzyme. Reactions were incubated at 37°C, and 10-\( \mu l \) aliquots were removed at 5, 15, and 30 min and stopped by adding to an equal volume of formamide dye solution (99% formamide, 5 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue). Typically, less than 10% of the primer was extended ensuring single cycle processive DNA synthesis. Reinitiation of DNA synthesis on an extended primer was not observed as evidenced by the lack of an effect of the time of incubation on termination probability. Reaction products were analyzed by 16% polyacrylamide gel electrophoresis, and the amount of radioactivity in the product bands.
FIG. 2. SDS-polyacrylamide gel electrophoresis analysis of HIV-1 mutant polypeptides. Photograph of Coomassie Blue-stained gel is shown. The location of the purified recombinant p66 polypeptides for wild-type (wt) and alanine mutant enzymes, and the top of the gel, are indicated on the right. Molecular weight markers (M) and their masses were: phosphorylase b, 97.4 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.3 kDa. The p66 subunit represents at least 90% of the total protein as determined from the integrated densitometry signals of each band measured using Millipore BioImage Visage Software. Some preparations of mutant enzyme also contained a small amount of p51 (<10%), which was assumed to form heterodimer.

was quantified using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). The termination probability for each position along the DNA template was calculated by dividing the number of product molecules of a given length by the sum of the number of product molecules of that length and the number of all longer products (9).

RESULTS

Alanine-scanning Mutagenesis—A collection of expression constructs was prepared in which residues 277-287 of the RT gene were individually modified to alanine. This region includes α of the thumb subdomain according to the structure of RT with bound DNA (3). Since alanine is indifferent to its location in α-helices (22), it serves as an ideal alternate residue to probe potential electrostatic and hydrophobic protein-DNA interactions. Each altered RT gene was expressed in Escherichia coli, and the recombinant enzymes were soluble in the crude cell extracts except for L283A and T286A. These two mutants were not characterized further. Following purification, SDS-polyacrylamide gel electrophoresis analysis indicated that the mutant RT polypeptides had the same apparent molecular weight as the wild-type polypeptide and were greater than 95% homogeneous (Fig. 2). The mutant p66 polypeptides represent, therefore, primarily homodimer preparations. Beard and Wilson (15) have demonstrated that the RT homodimer is kinetically indistinguishable from the heterodimer and can serve as a useful model for examining altered forms of the enzyme.

Deoxynucleoside Triphosphate Binding—When the dissociation rate constant for T-P (k\textsubscript{d}) does not limit k\textsubscript{cat}, then for the model illustrated in Scheme 1, K\textsubscript{m} and K\textsubscript{d,DTTP} are equivalent to K\textsubscript{S} and K\textsubscript{d,DTTP}. These kinetic parameters can, therefore, be estimated by measuring k\textsubscript{cat} and K\textsubscript{m} with dTTP and poly(rA)-oligo(dT)\textsubscript{20}. Fig. 3 (left panel) illustrates the results of K\textsubscript{m} determinations for dTTP. Alanine mutants of α\textsubscript{H} were similar to alanine mutants of α\textsubscript{H} (7) in that the K\textsubscript{m,DTTP} was generally unchanged relative to wild-type enzyme. The lack of a significant effect on dNTP binding also suggests that the alanine substitution does not globally perturb the structure of the altered enzyme, since α\textsubscript{H} is distant from the putative dNTP binding site.

Inhibition of dTTP incorporation with poly(rA)-oligo(dT)\textsubscript{20} by AZTTP can be a sensitive assay to probe polymerase and dNTP, as well as template-primer interactions (7, 20). Since AZTTP is a substrate, the K\textsubscript{i} determined when measuring the inhibition of dTTP incorporation is equivalent to the K\textsubscript{m} for AZTTP incorporation (Scheme 1), where K\textsubscript{m,AZTTP} = K\textsubscript{i}(k\textsubscript{cat}/k\textsubscript{d}) (17). The K\textsubscript{i} for AZTTP inhibition is, therefore, sensitive to K\textsubscript{d}, and AZTTP, template-primer dissociation rate constant (k\textsubscript{d}), and rate constant for nucleotide incorporation (k\textsubscript{cat}).

In contrast to several alanine mutants of α\textsubscript{H} (cf. Fig. 5 of Ref. 7), alanine mutation of α\textsubscript{H} did not change the sensitivity of the mutants to AZTTP (Fig. 3, right panel). Because K\textsubscript{i,AZTTP} is influenced by k\textsubscript{eff,T-P} and k\textsubscript{cat}, as discussed above, these results suggest that T-P binding (i.e. k\textsubscript{d}) as well as catalytic activity (i.e. k\textsubscript{cat}) should not be altered (see below). Although there are several potential electrostatic interactions between α\textsubscript{H}, of both the p51 and p66 subunits, and the sugar-phosphate backbone suggested by modeling studies (5, 6, 11), individual removal of these basic side chains appears to have little influence on apparent DNA binding affinity as revealed from the sensitivity of the mutant enzymes to AZTTP.

Template-Primer Interactions—To determine if template-primer interactions had been perturbed, the K\textsubscript{m} for poly(rA)-oligo(dT)\textsubscript{20} was determined. Consistent with the lack of an effect on sensitivity to AZTTP, alanine mutation of α\textsubscript{H} did not significantly alter the K\textsubscript{m,T-P} (Fig. 4, left panel). There was less than a 2-fold increase in K\textsubscript{m,T-P} for only one mutant of α\textsubscript{H} (Q278A), and two mutants (C280A and K281A) exhibited a 5-6-fold lower K\textsubscript{m,T-P}. Because binding of poly(rA)-oligo(dT)\textsubscript{20} is very tight, it is difficult to get an accurate estimate of the K\textsubscript{m,T-P} when the enzyme concentration is approximately equivalent to K\textsubscript{m,T-P}. Consequently, these small changes probably are not significant. In contrast to the small changes observed with the alanine mutants of α\textsubscript{H}, several alanine mutants of α\textsubscript{H} (Q258A, G262A, W266A) resulted in large increases in the K\textsubscript{m,T-P} (cf. Fig. 6 of Ref. 7).

To directly assess whether α\textsubscript{H} interacts with nucleic acid, the dissociation rate constant for the RT-T-P complex (k\textsubscript{d} or k\textsubscript{1} in Scheme 1) was measured. As demonstrated with α\textsubscript{H} (7), the dissociation rate constant for the RT-T-P complex is a sensitive index of the nature of the interactions between RT and T-P. The dissociation of the RT-T-P complex can be monitored by challenging the complex with heparin, to trap RT dissociating from T-P, and then assaying for the concentration of complex remaining after increasing periods of challenge (15). The dissociation rate constant for T-P with several alanine mutants of α\textsubscript{H} was increased modestly for several alanine mutants, but the increase was less than 4-fold when compared with wild-type enzyme (Fig. 4, right panel). The increase in k\textsubscript{eff,T-P} was generally observed when a basic side chain was replaced with alanine. T-P bound much weaker to G262A and W266A of α\textsubscript{H} relative to wild-type enzyme, as monitored by the dissociation rate constant for T-P (cf. Fig. 7 of Ref. 7).

Catalytic Activity—Whereas the activity (i.e. k\textsubscript{cat}) of the alanine mutants of α\textsubscript{H} were influenced differentially (cf. Fig. 3 of Ref. 7), the apparent activity of every mutant of α\textsubscript{H} was diminished, in one case 150-fold (L282A), relative to wild-type enzyme (Fig. 5). As noted above, this was unexpected since the K\textsubscript{i,AZTTP} (Fig. 3, right panel) for these mutants was not affected by the alanine substitution.
Mutagenesis of the Thumb Subdomain of RT

Since processivity is determined by kinetic competition between further extension ($k_p$) and dissociation of the enzyme from nucleic acid ($k_d$), termination probability is a measure of the ratio of these rate constants. The processivity of the alanine mutants of $\alpha_1$ was measured on M13mp2 DNA primed with a $^{32}$P-5' end-labeled 15-mer primer (8, 9). Primer extension reactions were performed using conditions that minimize reinitiation on previously extended primers (8–10, 23). Quantitative analysis of the probability of termination with the lacZ template indicated that the processivity of the alanine mutants of the basic residues of $\alpha_1$ was similar to wild-type enzyme (Fig. 6). Additionally, for all the other mutants of $\alpha_1$ altered by alanine substitution, there was no change in processivity as compared with wild type (data not shown). This is in contrast to G262A and W266A of $\alpha_1$, which had elevated $k_{cat,app}$, $K_{cat}$, and reduced processivity on the lacZ template (8).

The processivity measurements support the minimal effect observed on the dissociation rate constant for the RT-T complex observed for the $\alpha_1$ mutants, but also suggests a lack of an effect on $k_3$ as indicated by the sensitivity of these mutants toward AZTTP (Fig. 3, right panel).

Since $\alpha_1$ in the p51 subunit is near the subunit interface, the alanine mutation may be perturbing dimerization of the respective subunits leading to an apparent loss of activity. The dimeric concentration of enzyme would be overestimated from the total protein concentration, resulting in an underestimation of the catalytic rate (i.e. $k_{cat,app} < k_{cat}$). Since dimeric enzyme binds poly(rA) oligo(dT)$_{20}$ tightly and stoichiometrically (15), we titrated R284A (6% of wild-type activity) with T-P (Fig. 7, top panel). The titration indicates that all the protein in the reaction mixture can bind nucleic acid tightly ($K_d < 1 \text{ nM}$) and that this mutant enzyme is dimeric. Therefore, it is unlikely that the alanine substitution altered the concentration of dimeric RT. T-P titration of L282A (<1% of wild-type activity) gave similar results (data not shown).

An alternative explanation for the apparent lower activity is that alanine substitution results in a lower fraction of active enzyme. When product release is the rate-limiting step during
catalytic cycling, then a burst of product formation is observed upon initiation of the reaction (Scheme I, $k_4$, $k_3$). The amount of product formed during the burst should be equivalent to the concentration of active enzyme and has been observed previously (17–20). R284A has only 6% the activity of wild-type enzyme (Fig. 5). The active fraction of enzyme as determined from the burst of product formation on heteropolymeric DNA indicates that 9% of the mutant enzyme is active, relative to wild-type, and that $k_{obs, DNA}$ is 3-fold higher than with wild-type enzyme (Fig. 7, bottom panel), as observed with a RNA template (Fig. 4, right panel). Increasing the enzyme concentration gave a proportional increase in the burst amplitude (data not shown). It should be noted that the wild-type enzyme exhibited a burst amplitude 60% of that expected from protein determination and T-P titration. This is similar to the burst amplitude measured by Kati et al. (18) using rapid mixing and quenching techniques.

Fidelity in Forward Mutation Assay—The fidelity of the wild-type and mutant derivatives was examined in a forward M13mp2 gap-filling assay as described under “Experimental Procedures.” This assay scores 241 base substitution errors arising from possible mispairs at 125 sites within the 258 base $lacz_a$ gene target. In addition, single base frameshifts can be detected at 199 different sites as well as large deletions, duplications, and complex mutations. The results of the forward mutation assay yielded an average mutant frequency of $1.6 \times 10^{-2}$, consistent with previous reports for this enzyme (8). The forward mutant frequency of wild-type homodimer is the same as that observed for the heterodimer, but greater than that observed for most other DNA polymerases (14). As can be seen from Fig. 8, the mutants of $\alpha I$ produced $\alpha$-complementation mutants during DNA-dependent DNA synthesis with frequencies similar to wild-type. These frequencies are approximately 25-fold higher than background. Alanine substitution of $\alpha H$ resulted in several mutant enzymes that exhibited a lower fidelity than wild-type enzyme as measured by this forward mutation assay (7, 8). These mutants had lower fidelity for template primer slippage errors (8).

The overall average forward mutant frequency for the C280A derivative was elevated approximately 1.5-fold when compared with the wild-type RT. Because error rates are highly sequence context-dependent, we examined the error specificity of the $lacz_a$ complementation mutants generated in the forward assay. Fifty independent $lacz_a$ complementation mutants were isolated and sequenced to determine the distribution and types of errors produced by this derivative. These data were compared with a collection of 189 independent wild-type $lacz_a$ complementation mutants. No significant differences in quantitative error rates were found for both frameshift and base substitution errors, indicating the C280A derivative has a similar error specificity as the wild-type RT (data not shown).

DISCUSSION

Polymerase-Nucleic Acid Interactions—According to the crystallographic structure of HIV-1 RT complexed with DNA (3), two $\alpha$-helices (H and I) in the thumb subdomain interact 3–5 ($\alpha H$) and 6–9 ($\alpha I$) nucleotides from the polymerase active site with the primer and template strands, respectively (Fig. 1). These protein-nucleic acid interactions appear to occur in the vicinity of a bend in the DNA where a structural transition
from A- to B-form occurs 4–6 nucleotides from the 3′-primer terminus. Primary sequence comparison of this region, residues 254–288, with other nucleic acid polymerases revealed that limited sequence homology existed with a wide variety of RNA and DNA polymerases. This motif was suggested to act as part of a clamp in binding DNA and termed the helix clamp (6). Unfortunately, primary sequence homology could not be discerned for polymerases for which the crystallographic structure had been determined. Except for HIV-1 RT (3) and DNA polymerase β (24), there are no other polymerase-DNA complex structures in a “polymerization” mode. Although the DNA binding channel of DNA polymerase β is positively charged, only two basic side chains are within hydrogen bonding distance to the DNA, and these interactions are with bases within the DNA minor groove (24). No direct interactions are observed between any of the basic side chains of DNA polymerase β and the DNA backbone phosphates.

From examination of the 3.0-Å crystallographic structure (3), HIV-1 RT appears to make numerous protein-nucleic interactions in the vicinity of the polymerase active site. Interactions near the RNase H active site are deduced from enzymatic footprinting (11) and molecular modeling (5, 6, 11), since the duplex of the bound DNA (19-18-mer) is too short to deduce interactions with the distant RNase H domain. The distance between the polymerase and RNase H active sites within the DNA binding channel is approximately 15–19 nucleotides as determined by chemical footprinting (25) or kinetic coupling between polymerase and RNase H activities (18).

Interactions near the polymerase active site of the p66 subunit are contributed by the fingers, palm, and thumb subdomains. The 3′-OH at the primer terminus may be positioned by the β12-β13 hairpin (residues 227–235) and has been termed the “primer grip.” A “template grip” has also been described which interacts with the DNA sugar-phosphate backbone of the first four nucleotides of the template strand. These include β4 and aβ (residues 73–83) of the fingers subdomain and β5α (residues 86–90), β5a-β5b connecting loop (residues 91–93), and β8-αE (residues 148–154) connecting loop of the palm subdomain (3, 5).

Although the DNA in the crystallographic structure has only a single nucleotide overhang, important single-stranded template interactions have been suggested by site-directed mutagenesis analysis of the fingers subdomain (26). RT residues that have been implicated in HIV-1 viral sensitivity toward nucleoside inhibitors have been suggested to interact with the single-stranded template (4). Since nucleoside inhibitor sensitivity is dependent on T-P binding affinity, the lack of wild-type RT sensitivity to these inhibitors with short template overhangs (1–3 nucleotides) is consistent with a lower DNA binding affinity for DNA substrates with short single-stranded template overhangs (27). The low sensitivity of the mutant RTs (i.e. L74V and E89G) with longer single-stranded templates is consistent with a lower affinity of these polymerases for T-P (26).

In the thumb subdomain of RT, αH and αl appear to interact with the sugar-phosphate backbone. Alanine-scanning mutagenesis of αH has revealed that several residues on one side of αH interacts with DNA and substitution of these side chains with alanine drastically lowers the affinity of the polymerase for nucleic acid resulting in a lower in vitro sensitivity to AZTTP and processivity (7, 8). Additionally, two alanine mu-
Polymase Activity—Whereas nucleic acid binding was not influenced to an appreciable extent for the alanine substitutions in αI, the apparent activity of all the alanine mutants was significantly lower than wild-type enzyme (Fig. 5). This is in contrast to what would be expected from the sensitivity of these mutants to AZTTP (Fig. 3, right panel) and polymerase processivity (Fig. 6), since these parameters are sensitive to the magnitude of the rate constant controlling incorporation (k₄ in Scheme I). These results suggest that the active fraction has activity similar to wild-type enzyme, but that a population of enzyme in the reaction mixture is not active. One possibility we considered was that since active RT is dimeric, the alanine substitution may be altering the amount of dimeric enzyme and therefore lowering the apparent activity. However, titration of two alanine mutants expressing the lowest measurable activity with T-P indicated that the lower activity was not due to the inability of the mutant enzymes to form dimers since one T-P binding site per RT dimer was measured. Additionally, the dimeric enzyme was able to bind T-P tightly (K₄ < 1 nM; Fig. 7, top panel).

RT subunit dimerization has been suggested to occur in at least two steps (30). The first step forms the T-P binding site per enzyme. This is followed by slow structural rearrangements leading to active enzyme. To determine if the alanine mutants may represent a dimerization intermediate form, the active fraction of enzyme was determined from the magnitude of the burst of product formation occurring in the first turnover. When product release is slower than chemistry (k₄ << k₃, Scheme I), the amplitude of the burst represents the active fraction of enzyme (17–20). Time courses for the incorporation of a single nucleotide demonstrated that the burst amplitude for R284A was lower than wild-type enzyme to the same extent as observed for kcat,app (Fig. 5 and Fig. 7, bottom panel). When one calculates the turnover number for the linear steady-state portion of the time courses using the active enzyme concentrations determined from the burst, the mutant enzyme has a 3-fold higher activity than wild-type enzyme (kcat,app = 0.008 s⁻¹). In this case, kcat is limited by the dissociation rate constant for nucleic acid (k₃ in Scheme I) which was observed to be elevated 3-fold for a RNA template (Fig. 4, right panel). Alanine mutation of αI, therefore, appears to influence the concentration of inactive dimeric enzyme. Even in the case of wild-type enzyme, a population of inactive enzyme which could bind template or primer tightly was observed.

Since the alanine substitution occurs in both subunits of the homodimer. The thumb subdomain of the p66 subunit analogous to p51 (p51') forms part of the subunit interface near the RNase H domain of p66. It is tempting to suggest that the alanine substitution in the p51' subunit was responsible for the altered population of inactive dimeric enzyme, since these positions are near the subunit interface in the "mature" heterodimer. However, mutagenesis of a leucine repeat motif, residues 282–310 (31), indicated that these mutants also expressed very low activity (13). In this case, subunit-specific mutagenesis indicated that mutagenesis of Leu²⁸⁹ in p66, but not Leu²⁸⁹ of p51, abolished dimerization. The p66 thumb appears to be conformationally mobile. In one form of the apoenzyme it is in a closed conformation (32), and in the complex with DNA (3) or nevirapine (1), it is open. At the tip of the thumb in the closed form, Leu²⁸⁹ makes a hydrophobic contact with the fingers subdomain. Therefore, movements of the thumb subdomain of p66 may also play an important role in dimerization or enzyme "activation." It has been suggested that the monomeric subunits (p66 and p51) are in a compact conformation where the thumb is open and the connection subdomain is folded into the polymerase active site to remove hydrophobic surface area (33). Alterations in the p66 thumb subdomain which favor this compact structure requiring the thumb to be in an open structure may influence dimerization.

Conclusions—Although one face of αI of the thumb subdomain of HIV-1 RT is very basic, the putative interactions with the anionic DNA sugar-phosphate backbone are either very weak or kinetically silent. The predominant interactions occurring between RT and nucleic acid are with αH and the DNA minor groove. Alterations of these interactions in either RT (7, 8) or nucleic acid (9, 10, 34) decreases nucleic acid binding affinity and increases template primer slippage-initiated errors. Processive replicative polymerases should bind DNA tightly, but with low sequence specificity. Protein-nucleic acid interactions with the sugar-phosphate backbone are suitable for non-specific sequence recognition, but αH in the thumb subdomain of RT appears to offer very little binding free energy. DNA minor groove hydrogen bonding offers lower sequence specificity (35), but at the same time could offer tight binding if suitable nonpolar surfaces of αH are buried upon complex formation (36).

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