Insights into the role of an active site aspartate in Ty1 reverse transcriptase polymerization

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Summary

LTR-retrotransposons encode reverse transcriptases (RTs) that replicate their RNA into integratable, double-stranded DNA. A mutant version of the RT from *Saccharomyces cerevisiae* retrotransposon Ty1, in which one of the three active site aspartates has been changed to asparagine (D211N), is still capable of *in vitro* polymerization although it is blocked for *in vivo* transposition. We generated recombinant WT and D211N Ty1 RTs to study RT function and determine specific roles for the D211 residue. Pre-steady-state kinetic analysis of the two enzymes shows that the D211N mutation has minimal effect on nucleotide binding, but reduces the $k_{pol}$ by ~230 fold. The mutation reduces binding affinity for both Mn$^{2+}$ and Mg$^{2+}$, indicating that the D211 side chain helps create a tight metal binding pocket. Although both enzymes are highly processive and tend to remain bound to their initial substrate, each shows distinctive patterns of pausing, attributable to interactions between metal ions and the active site residue. These results provide insights to specific roles for the D211 residue during polymerization and indicate unusual enzymatic properties that bear on the Ty1 replication pathway.
**Introduction**

Reverse transcriptases (RTs) are DNA polymerases that can use either RNA or DNA as their template. These enzymes are essential in multiple biological settings, ranging from retroviral and retrotransposon replication to telomere maintenance. A characteristic feature of the primary amino acid sequences of RTs is the presence of the “YXDD motif” (1-3). The two invariant D residues, as well as a third D that is amino terminal to this motif, are positioned within the active site of the three RTs whose crystal structures have been solved (4-8). By analogy with other polymerases, which contain either two or three invariant Ds, it has been suggested that these residues function to coordinate the two catalytic metal ions involved in the polymerization reaction. Polymerization is a complex process and the architecture of the active site must properly position incoming dNTPs and the primer 3’ OH, aid in the nucleophilic attack, stabilize the transition state, assist in the removal of the pyrophosphate leaving group, and allow translocation of the primer end by one base. The active site D residues may influence all of these processes either directly or indirectly.

Among the myriad of RTs, HIV-1 RT has received the lion’s share of biochemical and genetic attention. Early mutagenesis studies indicated that substitution of any of the three aspartates led to loss of both infectivity and *in vitro* polymerase activity (9,10). Because of the severe biochemical defects associated with these substitutions, the consequences of modifications of these residues have not been extensively studied. Only one report measured steady-state biochemical parameters for mutant HIV RTs in which the first D (110) was changed to A or S, the second D (185) was changed to A, E or N, and the third D (186) was changed to A, E, or N (11). These changes resulted in a decrease of $k_{cat}$ by ~1 -10 thousand fold, indicating that
these modifications severely crippled but did not entirely eliminate the enzymatic activity of the mutant enzyme.

Ty1 is an LTR-containing retroposon of the yeast Saccharomyces cerevisiae. The genetic structure and the mode of replication of this element are, to a first approximation, identical to retroviruses. The main difference is that Ty1 is endogenous and noninfectious. We previously generated D to N or D to E mutations for each of the three invariant Ds in Ty1 RT. Examining mutant enzymes within Ty1 virus like particles (VLPs), we found that none were capable of in vitro polymerization except for D211N (equivalent to HIV-1 D186), using a standard homopolymer assay (12). Characterization of VLP contents showed that the D211N mutant could polymerize limited amounts of minus-strand strong stop DNA and strand transfer, but we could not detect plus-strand DNA within mutant VLPs. Genetic tests showed that while this mutant was incapable of transposition, second site mutations in the Ty1 RNase H domain could suppress the transposition defect of D211N and restore transposition to ~5-10% of WT levels. These studies suggested that while the aspartate side chain at position 211 was not essential for the singular act of polymerization, it was required for other steps in the replication process (12).

Sequence alignments of a wide range of polymerases, as well as comparisons of multiple polymerase crystal structures, shows that only two of the three active site carboxylates are conserved (13,14). A specific role for the third D (equivalent to Ty1 D211) found in all RTs has not been established. Given that a likely function of the active site carboxylates is coordination of two catalytic metal ions an understanding of the interactions between the aspartate side chain, the metals, and the other components of the active site, is of great interest. The ability of the Ty1 RT D211N mutant to retain polymerase activity, despite substitution of a critical residue, indicates
that this altered enzyme can be used to better understand the catalytic function of the active site D residues in RTs.

While VLPs are excellent sources of Ty1 intermediates, they are too impure to use for detailed biochemical comparisons of RT, as they contain multiple other activities at stoichiometric amounts. We have therefore purified recombinant forms of WT and D$_{211}$N RT and compared their pre-steady-state or steady-state parameters associated with a number of steps in polymerization, including nucleotide binding, single base addition, divalent metal ion binding, and dissociation of the enzyme from the template. Since most of these parameters have not previously been studied for the WT enzyme, this allowed us to compare the retrotransposon enzyme to retroviral RTs, as well as to look at the specific effects of this novel active site mutation. A previous study comparing a different recombinant form of WT and D$_{211}$N Ty1 RT, concluded that the WT enzyme binds Mg$^{2+}$ with high affinity but Mn$^{2+}$ with very low affinity, while the mutant enzyme behaves in a reciprocal manner (15). On the contrary, we find that the mutant enzyme has reduced affinity for both catalytic metals, and that the two different metals affect polymerization of the two enzymes in idiosyncratic concentration-dependent ways. By single base turnover analysis, we find that the D$_{211}$N substitution has an effect on the rate of polymerization, indicating a role in the chemistry of polymerization, but that it is not involved in either dNTP binding or dissociation of enzyme from its primer/template. Finally, the D$_{211}$N mutant results in distinctive changes in the pattern of pausing during polymerization, suggesting a role for this residue in pyrophosphate release and/or translocation.

**Experimental Procedures**

**Plasmids and Strains**
The plasmid p6H Ty1 IN-RT-RH (construct 2 in (16)) contained WT Ty1 RT–RH plus a 115 amino acid contiguous C-terminal portion of Ty1 integrase fused to the N-terminus of the RT-RH domain, all preceded by 6 histidine residues. It was kindly provided by Dr. F. X. Wilhelm (IBMC, Strasbourg) and designated AGE2186 in our laboratory. These extra 115 amino acids are part of a C-terminal extension found in Ty1 integrase, which is far downstream of all potential metal binding domains of Ty1 integrase (17). The fusion construct was used because equivalent recombinant proteins beginning at the N terminus of the RT-RH domain totally lack in vitro polymerase activity ((16) and A. Gabriel, unpublished results). We had previously constructed the D211N polymerase active site mutation and expressed it in plasmid pGTy1D211Nmhis3AI (AGE1603) (12). To make the analogous mutant expression plasmid, constructs AGE1603 and AGE2186 were cut with Sph I and Afl II and the 386 bp fragment of the former was ligated to the 5.016 kb fragment of the latter, to generate AGE2352 in strain XL1-Blue. The WT and mutant plasmids were transformed into E. coli expression strain M15 containing pREP4 (Qiagen) to generate AGE2193 and AGE2354 respectively. A detailed list of reagents and protocol for expression and purification of recombinant Ty1 RT are in the supplementary information. Note that the only difference between the two enzymes is the presence of D or N at position 211, and that the two enzymes are expressed at similar levels in E. coli.

**Primer Extension Assay with DNA Templates**

Procedures were modified from those of Patel et al (18). In the usual assay, a DNA/DNA primer/template was prepared by annealing a 28-mer plus-stand sequence from the polypurine tract region of Ty1 RT with a complementary 14-mer primer (primer/template ratio is 1/0.85), whose 5'-end is 32P-labeled, to generate the following model substrate:
Detailed descriptions of the primer extension conditions, labeling of oligonucleotides and DNA markers and denaturing PAGE are in the supplementary information.

**Homopolymer assay for RT**

Routine RT assays during purification were carried out by estimating the dGTP incorporation into poly r(C)/oligo d(G)\textsubscript{14-18} using the filter paper binding method (16).

**Determination of $K_d$ and $k_{pol}$ for the incorporation of $dATP$**

The pre-steady-state kinetic parameters for single correct base incorporation by the WT and the mutant enzymes were measured as reported by Patel et al. (18) with the following modifications. Reactions were carried out by rapid quench analysis (19) for WT using a KinTek RQF-3 Rapid Quench Flow apparatus, and manually for the mutant. Protein concentration for both enzymes were determined at OD\textsubscript{280} in a 1 cm cell in the presence of 8 M urea, (extinction coefficient for the 76.861 kDa protein = 86,390/cm M). Enzymes were used at final concentrations of 228.3 nM (WT) and 92.64 nM (mutant), assuming that the enzyme is a monomer (F.X. Wilhelm, unpublished results) which were equivalent to active concentrations of 15.97 nM for WT and 15.66 nM for D\textsubscript{211}N as determined by 3 minute reactions at 200 micromolar dATP. Analysis was performed using a 5\textsuperscript{′} 32\textsuperscript{P}-end labeled 14-mer/28-mer substrate, as above, in the presence of varying amounts of the single nucleotide dATP. For WT, solution one (final concentrations of 40 nM primer/template with RT) was prepared in extension buffer (17 mM Tris-HCl (pH 7.5), 17 mM NaCl, 1 mM dithiothreitol and 20% glycerol) and loaded in one of the syringes. Solution two contained extension buffer with the appropriate [dATP] and MgCl\textsubscript{2} to a final concentration...
of 20 mM and was loaded in the other syringe. Reactions were carried out at 22°C by mixing equal volumes. Samples were taken at various time points and quenched with EDTA, at a final concentration of 35 mM. For D$_{211}$N mutant Ty1 RT, reactions were carried out manually with similar solutions one and two after 10 minutes of pre-incubation of enzyme with substrate at 22°C. Samples were taken at intervals over 4 minutes, and immediately quenched with the loading buffer (95% formamide, 35 mM EDTA [pH 8], 0.1% bromphenol blue, 0.1% xylene cyanol). Products were denatured and resolved in 17% denaturing gels, scanned and then analyzed. The time course of a single turnover for individual [dATP] was fitted to an exponential equation (Eqn. 1, see supplementary information). The calculated rates were plotted against respective [dATP] to fit this data to a hyperbolic equation (Eqn. 2, see supplementary information).

**Determination of the $K_d$ of the divalent ions**

Reactions were carried out in the presence of various concentrations of divalent ions, 0 - 230 mM Mg$^{2+}$ (as MgCl$_2$) and 0 - 49 mM Mn$^{2+}$ (as MnCl$_2$), in a 10 minute extension assay using the 5'-$^{32}$P-end labeled 14-mer/28-mer substrate, with 8.0 micromolar each dNTP and equivalent amounts of active WT or D$_{211}$N enzymes. Extended samples were resolved on gels and analyzed, as above. Amount of metal ion bound to dNTP at each total metal ion concentration was calculated using a quadratic equation (Eqn. 3, see supplementary information). For this estimate, we used a $K_{d\text{Mg-dATP}}$ of 20 micromolar (20) and a $K_{d\text{Mn-dATP}}$ of 9.77 micromolar (21). The amount of product formed versus the free metal ion concentration was fitted to the Hill equation (Eqn. 4, see supplementary information).
Pausing experiments

We used the same 5’ ³²P-end labeled 14-mer/28-mer DNA/DNA substrate (40 nM) for this experiment. All reactions were performed in extension buffer at the specified concentrations of Mg²⁺ or Mn²⁺ using WT or D₂₁₁N enzymes. The reactions were divided into two, without or with an excess of an activated calf thymus DNA trap at 2.63 mg/ml, final concentration. Reactions were initiated by adding all four dNTPs (8.0 micromolar each), to the pre-warmed enzyme and labeled DNA substrate. The reactions were incubated at 22°C for 10 minutes. All reactions were stopped with loading buffer, and denatured samples were analyzed by electrophoresis as described. A trap effectiveness control was also carried out where the reaction was initiated by adding dNTPs with Mg²⁺ or Mn²⁺ to a mix containing enzyme, trap and primer/template (where enzyme was added to the labeled substrate in the presence of trap and then pre-warmed along with the two). For the DNA/RNA substrate, we used the 5’ labeled 14-mer DNA primer as above and a 28-mer RNA template, whose sequence was equivalent to the above DNA template: (5’-AUU ACA UUA UGG GUG GUA UGU UGG AAU A -3’). These reactions were also carried out in the presence and the absence of trap. However, here we used the unlabeled DNA/DNA substrate as a trap at 200-fold excess to the labeled substrate concentration. Reactions were carried out at 10 or 30 mM Mg²⁺ or at 2 mM Mn²⁺.

Processivity Assay

Poly r(A) (500 - 600 nt) or poly d(A) (350 – 400 nt) primed with 5’ end labeled oligo d(T)₁₄-₂₈ oligonucleotide (0.0894 ng/microliter final concentration) was pre-incubated with WT or D₂₁₁N Ty1 RT for 10 minutes at 22°C. The extension reactions were initiated by adding a mixture containing 24 micromolar dTTP along with 10 mM or 30 mM Mg²⁺ or 2 mM Mn²⁺ with or without an unlabeled 14-mer/28-mer DNA/DNA substrate trap (at a final concentration of 200
fold more than the labeled primer/template). Reactions were carried out in the extension buffer at 22°C for 30 minutes and then terminated with loading buffer. Control reactions were carried out using Klenow polymerase under the same reaction conditions, with 10 mM Mg\(^{2+}\) or 2 mM Mn\(^{2+}\). Trap effectiveness controls were carried out as above. The terminated processivity reaction and control reactions were resolved by 6% polyacrylamide-urea denaturing gel electrophoresis and analyzed as above.

**Measurement of the Dissociation Rate for the Ty1 RT•DNA Complex**

Ty1 RT (18 nM of active WT or mutant enzyme) and 40 nM 5' labeled 14mer/28-mer were combined in a reaction mixture containing Mg\(^{2+}\) (final concentration, 10 mM) and then pre-incubated in the extension buffer for 10 minutes to allow the enzyme•DNA complex to reach equilibrium. A 200-fold excess of unlabeled substrate was then added to the reactions as trap for the dissociated enzyme. At various time points after the addition of the trap (0 to 180 minutes) samples were withdrawn, and mixed with the next correct nucleotide, present in large excess, to initiate DNA synthesis for 3 minutes. Control reactions with Moloney murine leukemia virus (MMLV) RT was also conducted using the same substrate but at 50 mM Tris-HCl (pH 7.5), 76 mM NaCl, 10 mM Mg\(^{2+}\), 0.05% NP-40, 20% glycerol, and 20 mM DTT [Roth, 1985 #546]. Trap effectiveness reactions were performed by adding the enzyme to the mix of labeled and unlabeled substrates, pre-incubated for 10 minutes, then the reaction was started by adding dATP and Mg\(^{2+}\) for 3 minutes. For the 0 time samples, dATP and Mg\(^{2+}\) were added along with the trap and reaction was carried out for 3 minutes. The reactions were terminated with loading buffer and the samples were analyzed on denaturing gels. The amounts of the extension products (15-mer and greater) were determined relative to total labeled product (extended and un-extended 14-mer). These values were normalized for 0 seconds, and then the ratio of intensity of extension
products/total intensity were plotted against the time in minutes. The obtained curves were fitted to a single exponential equation using a three parameter fit for the decay (Eqn. 5, see supplementary information).

Results

Pre-steady-state kinetics of dATP incorporation of WT and mutant D_{211}N Ty1 RT

Recombinant versions of both WT and D_{211}N mutant Ty1 RT enzymes were purified by nickel-affinity chromatography (Fig. 1). Both WT and D_{211}N mutant enzymes were active in a standard homopolymer assay, using α-[^{32}P] dGTP (data not shown), as expected from previous studies (12,15). The homopolymer assay is, however, extremely crude and not designed to measure subtle differences between enzymes.

As a baseline for comparing the polymerization properties of the WT and D_{211}N enzymes, we therefore used a model substrate and measured the $K_d$ and $k_{pol}$ for single nucleotide incorporation for the two enzymes, using pre-steady-state kinetics, based on the following minimal scheme for single base addition:

\[
E + T \cdot P_n \xrightleftharpoons{k_{\text{off}}} E \cdot T \cdot P_n + dNTP \xrightarrow{k_{\text{on}}} E \cdot T \cdot P_n \cdot dNTP \xrightarrow{k_{\text{pol}}} E \cdot T \cdot P_{n+1} + PP_i
\]

To this end, a 5’ end-labeled 14-mer primer was annealed to a 28-mer template, mixed with RT, synthesis was initiated by the addition of varying concentrations of dATP in the presence of Mg^{2+} and reactions were quenched at various times. Labeled products were visualized and quantitated (see Experimental procedures). The single exponential equation (Eqn. 1, in supplementary information) fit the kinetics well, as shown by the solid lines in Fig. 2 a & b. The derived exponential rate constants were plotted against their respective dATP concentrations and the curve was fitted to a hyperbolic equation (Eqn. 2 in supplementary information and Fig. 2 c)
& d) from which $K_d$ and $k_{pol}$ values were derived. In deriving $K_d$, we assumed that dNTP is in rapid equilibrium with $E \cdot T \cdot P_n$ (see Scheme).

The Table shows $K_d$ values of 2.4 ± 0.4 micromolar and 1.0 ± 0.4 micromolar and $k_{pol}$ values 7.8 ± 0.4 sec$^{-1}$ and 0.035 ± 0.002 sec$^{-1}$ respectively for the WT and mutant D$_{211}N$ enzymes. Since the $K_d$ provides a measure of enzyme affinity for dATP, it appears that the WT and D$_{211}N$ RTs have similar substrate affinity. In contrast the D$_{211}N$ mutant showed a large defect in the rate of polymerization (226 fold less) relative to WT. The derived catalytic efficiency, i.e. the ratio of $k_{pol}$ and $K_d$, was ~100 fold lower for the mutant RT enzyme. These results indicate that the D to N substitution of the third aspartate in the catalytic triad does not interfere with the binding of the deoxynucleoside triphosphates at the active site. Instead the mutant enzyme carries out the polymerization step at a reduced rate. The replacement of D at position 211 by N reduces rates of one or more steps of the polymerization reaction by a factor of 226, implying that this residue has a significant role in optimizing the chemistry of polymerization.

**Primer extension patterns differ for WT and D$_{211}N$ mutant RTs on a model substrate**

We next compared the extension pattern of the two enzymes on the same model 14-mer/28-mer DNA/DNA substrate, in the presence of all four dNTPs. First we examined the pre-steady-state rates of extension for multiple base additions, using saturating concentrations of dNTPs. The results were similar to those obtained with our single base addition studies (data not shown). Next we compared the extension patterns of the two enzymes in the presence of either MgCl$_2$ or MnCl$_2$. The two enzymes generated distinct extension patterns under these conditions (Fig. 3). In the presence of 10 mM Mg$^{2+}$, the WT enzyme was able to fully extend the primer within 10 minutes (Fig. 3, lane 5), and further extension consisted of non-templated base addition beyond
the 28-mer length (Fig. 3, lane 7). For the D_{211}N RT, extension was slower (Fig. 3, lanes 6 and 8). In the presence of 100 micromolar Mn^{2+} the WT enzyme was also able to extend to the end of the template, but as opposed to the situation with Mg^{2+}, several intermediate bands were seen. This suggests either stalling or dissociation of enzyme from primer/template (Fig. 3, lanes 9 and 11). We refer to this phenomenon as “pausing”. The D_{211}N RT carried out more complete extension in Mn^{2+} than in the presence of Mg^{2+}, and has distinct pause sites compared with Mg^{2+} (compare Fig. 3, lanes 8 and 12). Nontemplated base addition was much more apparent in the presence of Mn^{2+}, particularly for the WT enzyme, where up to 4 bases were added beyond the template.

**D_{211}N enzyme has reduced binding affinity for divalent ions**

To see how the concentration of Mg^{2+} or Mn^{2+} affected the extent and pattern of the polymerization reaction, we carried out primer extensions in the presence of a wide range of [Mg^{2+}] or [Mn^{2+}] (Fig. 4). The WT enzyme was capable of full-length extension plus nontemplated extension, without pauses, through a wide range (~0.080 mM to ~200 mM) of [Mg^{2+}] (Fig. 4 a). At the highest and lowest [Mg^{2+}], nontemplated base additions were not observed. Full-length extension with the D_{211}N RT occurred over a much narrower range of [Mg^{2+}] (~5 to ~151 mM, under these conditions) and distinct pausing bands were seen at different divalent metal ion concentrations (Fig. 4 b). Extension was only apparent for the mutant enzyme at Mg^{2+} concentrations above 0.156 mM, > 300 fold higher than was seen with the WT enzyme. At the lowest and highest [Mg^{2+}], extension did not proceed to the end of the template in the 10 minutes time span of the reaction.

Figs. 4 c and 4 d show a very different pattern of primer extension for these two enzymes in the presence of Mn^{2+} instead of Mg^{2+}. Full-length extension of the WT enzyme is reduced at
[Mn\(^{2+}\)] > 0.024 mM and significant levels of full-length extension only accumulated within a narrow range of [Mn\(^{2+}\)] (0.006 – 0.024 mM, Fig. 4 c) during the 10 minute reaction.

Interestingly, the overall proportion of product that is extended in WT reactions does not decrease as a function of higher metal ion concentration. However, at [Mn\(^{2+}\)] above 0.048 mM, most products were extended by a single base, and the proportion of full-length products decreased. Further, three distinct pausing patterns were observed at different [Mn\(^{2+}\)]. Each of these patterns was distinct from the extension pattern seen in the presence of Mg\(^{2+}\), and differed as well from those seen with the mutant enzyme under the same metal conditions.

For the D\(_{211}\)N enzyme, full-length extension products are seen only at [Mn\(^{2+}\)] ≥ 0.096 mM (Fig. 4 d). Distinct from the WT enzyme, no inhibition was observed at high metal ion concentrations. Extension products were first observed at a [Mn\(^{2+}\)] ~30 fold higher than that seen with WT enzyme (0.012 mM Mn\(^{2+}\) versus 0.00037 mM Mn\(^{2+}\)). To estimate the \(K_d\) for metal binding for the WT and D\(_{211}\)N RTs we plotted the overall extension data against the [Mn\(^{2+}\)]\(_{\text{free}}\), taking into account the metal binding of dNTP. In each case, the data were best fitted to the Hill equation (Eqn. 3 in supplementary information), and Hill constant values of ~2 were calculated, indicating a positive cooperativity of metal ions (Table). The D\(_{211}\)N enzyme had metal dissociation constant values ~91 fold and ~36 fold greater than the WT enzyme for Mg\(^{2+}\) and Mn\(^{2+}\) respectively, indicating that the N substitution generally reduces metal binding affinity.

WT \(K_{d,\text{Mg}}\) was ~21 times greater than \(K_{d,\text{Mn}}\) and D\(_{211}\)N \(K_{d,\text{Mg}}\) was ~53 times greater than that of \(K_{d,\text{Mn}}\), indicating that for both enzymes Mn\(^{2+}\) is more efficiently bound than Mg\(^{2+}\). Our findings suggest that the amide side chain in the mutant perturbs the geometry of the metal binding portion of the active site, thus allowing for weaker binding of either metal ion.

**Stalling on- versus dissociation from- the template**
Since both the WT and mutant enzymes showed distinct patterns of intermediate length products under different conditions, we next examined whether these patterns represent a problem of processivity, i.e. the enzyme falls off the primer/template and then has to restart synthesis, or whether they represent a problem of stalling, i.e. slowed processive synthesis along the template. We carried out processivity experiments to determine whether the presence of an excess of unlabeled trap substrate would affect the extension pattern of a pre-annealed enzyme•substrate complex. Various [Mg$^{2+}$] or [Mn$^{2+}$] were used to analyze the patterns of bands we previously observed under different metal ion concentrations. In the presence of Mg$^{2+}$ (10 mM-200 mM) extension patterns for the WT enzyme were unaffected by the presence of the trap (Fig. 5 a, lanes 1 through 6). For the mutant enzyme, a small decrease in full-length products was observed at 10 and 20 mM Mg$^{2+}$ (Fig. 5 a, lanes 9 through 12). When 2 mM Mn$^{2+}$ was used in the reactions both enzymes had the same extension patterns in the presence or absence of the trap (Fig. 5 b). Thus it appears that the observed pausing patterns are not primarily due to dissociation of the enzyme from its primer/template. Note that we used a Mn$^{2+}$ concentration in the millimolar range for this experiment, after we discovered that the trap chelated the metal in the more optimal micromolar range (see Fig. 4).

Since a DNA/RNA substrate is a natural substrate for RTs, we also compared the extension and pausing patterns of our 14-mer/28-mer DNA/DNA substrate with a sequence-equivalent DNA primer/RNA template. This allowed us to determine if pausing is a consequence of the chemical make-up of the template or depended more on the specific base sequence of the template. As shown in Fig. 6, lanes 5 through 12 and 21 through 28, the pausing patterns for each enzyme in the presence of Mg$^{2+}$ were similar, regardless of the type of substrate species used. In the presence of Mn$^{2+}$ however, the pausing patterns for both enzymes varied
depending on whether the substrate was DNA/DNA or RNA/DNA (lanes 13 to 16 for WT and 29 to 32 for the mutant). Further, both enzymes were less processive in the presence of a DNA/RNA substrate and Mn\textsuperscript{2+}, as shown by the reduced product size for WT and the reduced proportion of full-length extension product for the D\textsubscript{211}N mutant enzyme in the presence of trap. This suggests that enzyme binding to its substrate in the presence of an RNA template and Mn\textsuperscript{2+} is less tight than under other conditions.

**Reduced extension capacity for the D\textsubscript{211}N mutant enzyme with long templates**

Since extension by 14 bases is not a particularly long template upon which to judge processive polymerization, we next examined extension patterns using substrates consisting of poly r(A) or poly d(A) templates, primed with a 5'\textsuperscript{32}P-labeled oligo d(T)\textsubscript{14-28} oligonucleotide. Fig. 7 shows the resolved extension products in the presence or absence of trap. In the presence of Mg\textsuperscript{2+}, the WT enzyme was much more processive than Klenow (compare Fig. 7, lanes 5 through 8, with lanes 25 through 28) extending nearly to the lengths of the templates, regardless of the presence of the trap. For the mutant enzyme, the average product length was significantly shorter than WT in the presence of Mg\textsuperscript{2+}, although there was no evidence of enzyme dissociation from the substrate during synthesis. In the presence of Mn\textsuperscript{2+}, the WT enzyme showed a relative defect in the length of extension of a DNA/DNA substrate (Fig. 7, lanes 9 & 10), compared to the RNA/DNA substrate (lanes 11 & 12), or the D\textsubscript{211}N mutant with the same substrates (lanes 21 to 24) although extension is still trap-resistant.

As seen with the 14-mer/28-mer substrate, the WT enzyme extends well in the presence of Mg\textsuperscript{2+}, but has a relative extension defect in the presence of Mn\textsuperscript{2+}, particularly for DNA/DNA substrates. In Mg\textsuperscript{2+}, the WT enzyme appears extremely processive, extending over 600 bases in the presence of a trap (Fig. 7, lanes 1 to 8). The mutant enzyme extends poorly in the presence
of Mg$^{2+}$ but remains processive (lanes 13 through 20). Processive extension is clear in the presence of Mn$^{2+}$ (lanes 21 through 24) and does not discriminate between RNA and DNA templates. Both versions of the Ty1 enzymes are more processive than the Klenow fragment of \textit{E. coli} DNA polymerase I in the presence of either metal ion.

\textbf{Rate of Dissociation of the Ty1 RT•DNA complex is low}

To see if the mutant enzyme’s low $k_{pol}$ and unexpectedly high processivity are related to the rate of dissociation of enzyme from its DNA substrate, we measured the $k_{off}$ for both enzymes from the 5’ labeled 14-mer/28-mer DNA/DNA substrate, by incubating substrate and enzyme in the presence of a trap for various times before initiating extension. The resolved extension products were quantified, and the decreasing fraction of enzyme still bound to the DNA template ($I_{\text{extended}}/I_{\text{total}}$) was plotted versus time and $k_{off}$ for each enzyme was determined (Fig. 8). As shown in the Table, the rate of dissociation displayed by the mutant enzyme was ~3 fold slower than the WT enzyme (0.063±0.006 per min for WT and 0.022±0.003 per minute for the mutant D$_{211}$N RT). A similar $k_{off}$ determination for MMLV RT was roughly estimated, since in this case most dissociation had occurred by 0.5 minute, the shortest time point taken here. As shown in the Table, the retroviral enzyme dissociates from its template ~ 87 times more quickly than the WT and ~250 times than the mutant Ty1 enzyme.

\textbf{Discussion}

We have carried out the first pre-steady-state analysis of a retrotransposon RT, to better understand the biochemical properties of this enzyme and the defects associated with an unusual polymerase mutant. Although the D$_{211}$N substitution renders the mutated Ty1 element incapable of \textit{in vivo} transposition, \textit{in vitro} polymerization with homopolymeric templates is still observed (12,15). Further, second site substitutions in the RNase H domain restore 5 – 10 \% of the
transposition activity (12). Similar active site substitutions for HIV-1 RTs have yielded severely crippled enzymes with no infectivity (9-11,22). Second site suppressors of equivalent HIV-1 mutant have not been reported. Among other polymerases, substitution of active site carboxylates is also generally inactivating (23,24), but in a few cases, some level of activity can be restored in the presence of alternate cations, e.g. Mn$^{2+}$ (25,26).

Using pre-steady-state single turnover kinetic analysis we determined the dissociation constant ($K_d$) for dATP and the rate of the phosphoryl transfer reaction ($k_{pol}$), for both WT and mutant RTs. Both kinetic constants for the WT Ty1 enzyme (Table) are similar to those obtained for HIV-1 RT, by either pre-steady-state or steady-state calculations (11,27). Interestingly, although the $k_{pol}$ for the D$_{211}$N mutant was significantly decreased, the $K_d$ was similar to WT. Current models of polymerization suggest that dNTPs enters the active site already bound to a divalent metal ion (referred to as the B metal), and that this metal could also aid in expelling the PP$_i$ leaving group after the chemical step of catalysis (28,29). Our result suggests that the D$_{211}$ side chain is not crucial for entry of the dNTP into the binding site or for the formation of the coordination shell around the entering metal, consistent with the crystal structure of HIV-1 RT and other polymerases (7,28,30), as well as biochemical analysis of HIV-1 active site mutants (11). Based on these reports, positioning of the incoming dNTP•metal chelate is more likely a role for the D$_{210}$ and D$_{129}$ residues.

Given the large reduction in catalytic efficiency for the mutant enzyme, D$_{211}$ is appears to be involved in the catalytic steps of polymerization. Models of polymerization (31) propose multiple steps after formation of the ternary enzyme•substrate•nucleotide complex, including the conformational change to a transition state, chemical bond breakage and formation, the conformational change from the transition state to a ground state, followed by PP$_i$ removal and
primer end translocation. The D$_{211}$ side chain is likely to interact with metal ions, either directly or indirectly through a water molecule, given the very different response to Mg$^{2+}$ and Mn$^{2+}$ seen with the WT and mutant enzymes, which differ by only the single negatively charged side chain carboxylate versus the uncharged carbonyl amide. Mg$^{2+}$ has a slightly smaller ionic radius than Mn$^{2+}$ and is a “harder” metal than Mn$^{2+}$, much less likely to coordinate with an uncharged nitrogen compared with a negatively charged oxygen. Both metals coordinate readily with water or with carboxylate side chains, and prefer a coordination number of 6 (32,33). We found that both enzymes have a much lower $K_d$ for Mn$^{2+}$ relative to Mg$^{2+}$ (Table). Further, both metals bind tighter to the WT enzyme than to the mutant enzyme. Interpretation is complicated by the fact that two metal ions bind cooperatively in the active site, but the implication is that the D$_{211}$ side chain plays a role in creating a tight binding pocket for whichever metal is present.

The metal ion titrations (Fig. 4) showed striking differences in the patterns of metal-dependent primer extension. The WT enzyme is active over a very wide range of [Mg$^{2+}$] but has only a narrow range of [Mn$^{2+}$] in which full-length extension occurs (6–24 M). Above ~50 micromolar, Mn$^{2+}$ has an inhibitory effect on processive polymerization, causing the WT enzyme to stall after a single base addition (Fig. 4 c). The D$_{211}$N mutant has no such defect, and full extension occurs at levels of Mn$^{2+}$ as high as 50 mM. This indicates a concentration-dependent polymerization block resulting from interaction between the carboxylate side chain in the WT enzyme and the larger, more flexible Mn$^{2+}$. We propose that the chemical step of polymerization does occur, but a subsequent step (pyrophosphate release or primer-end translocation) is inhibited at saturating concentrations of Mn$^{2+}$. Thus the D$_{211}$ residue may have a role in these post-chemical steps. For the D$_{211}$N mutant enzyme, Mn$^{2+}$ behaved as a more functional metal than Mg$^{2+}$. The less favorable association between the Mg$^{2+}$ and the carboxyamide side chain in
the mutant enzyme may result in suboptimal coordination and reduced polymerization potential. The concentration-dependent inhibition of full-length extension by the mutant enzyme above 67 mM Mg$^{2+}$ corresponds with the loss of blunt end addition activity in the WT enzyme. These changes at high metal concentrations could reflect the fact that metal ions do not just bind in the active site and to the dNTPs, but non-specifically to other sites on the protein, as well as to the backbone phosphates of the primer and/or template (34).

Our finding that WT enzyme extension is blocked by Mn$^{2+}$ while mutant enzyme extension is inhibited by Mg$^{2+}$, are partially consistent with the findings of Bolton et al. (15). However, their analysis of metal ion dependence for WT and D$_{211}$N Ty1 RTs using a homopolymer substrate, differed in both absolute and relative terms from our measured $K_d$ values. In particular, their calculation of the $K_{0.5}$Mn$^{2+}$ for WT enzyme is 1000 fold higher than their $K_{0.5}$Mn$^{2+}$ value for the mutant. We found the WT enzyme $K_d$ for Mn$^{2+}$ to be 35 fold lower than the mutant, and in the range of 0.2 micromolar. The basis for the discrepancy between the two sets of data is not clear, but several experimental factors are different. The enzyme used by Bolton et al. contains a large GST amino terminal fusion, while ours contains a small hexahis tag. The subtle consequences of these appendages are unknown. The lower limit of the values tested by Bolton et al for the WT enzyme in the presence of Mn$^{2+}$ is, according to our Fig. 4, in the range where polymerization is blocked beyond the first base addition, well above the optimal range in which polymerization occurs. The concentration of dGTP that they used is 10 fold lower than our estimated $K_d$ for nucleotide binding, which could be another limiting factor. Further, Bolton et al measured % incorporation with a homopolymer substrate and did not resolve their products, whereas we measured the amount of extension using a specific substrate.
and a known amount of active enzyme in a time frame before reagents became exhausted, and visually determined the lengths of extension.

An unexpected finding of our study was the distinct patterns of pausing seen with the two enzymes under different metal conditions. Using traps we demonstrated that intermediate extension products were not due to enzyme dissociation. Both enzymes bind their primer/templates much more stably than either AMV or MMLV RTs, and had off rates similar to the RT from the non-LTR retrotransposon R2Bm (35). We also inferred tight binding by the processivity of the yeast enzymes (Fig. 7) as well as the relative lack of turnover seen during time courses (Figs. 2, 3 and data not shown). Instead of dissociating from the full-length extension product and associating with additional substrate, the major consequence over time for full-length WT enzyme extensions was an increase in nontemplated base additions (Fig. 3). This could explain our earlier finding of wide spread nontemplated base additions at the termini of double-stranded Ty1 DNAs (36). In vivo, stable RT binding to blunt double-stranded DNA ends could help recruit integrase to these ends, or protect the ends from nucleolytic degradation. If tight RT binding also occurs at the ends of intermediates during Ty1 replication, it could serve as an aide to strand transfer or to ensure that RNase H cleavage of sub-terminal sites is carried out.

Pausing has been previously observed during in vitro analysis of a number of polymerases. Klarmann et al. (37) correlated HIV pause sites with homopolymeric nucleotide runs and sites of potential hairpins in the template sequence. In their study pausing sometimes represented dissociation events whereas in other cases it represented slowed polymerization. We saw distinct pausing patterns with the WT and mutant enzymes, as well as with different concentrations of metal ions. Further, the pausing patterns differed in the presence of Mn$^{2+}$ if DNA or RNA served as the template. Almost all pause sites were independent of the presence or
absence of trap, indicating that they were not due to dissociation. The pausing pattern was sequence specific, since a different template of similar size gave different pause patterns (unpublished data). Pausing could be due to a relative block at many steps in the polymerization process. Analyzing the positions of different pauses, there was no obvious correlation of specific pause sites with either form of the enzyme or with the metal used. Instead it was clear that pausing did not occur in the WT enzyme in the presence of a wide range of [Mg$^{2+}$]. Since this is presumably the preferred metal used by the WT enzyme, it suggests that pausing is a function of distortion within the active site, either because of the N side chain in place of D, or because of the imperfect fit of Mn$^{2+}$ as the chelated catalytic ion.

Our biochemical analysis of the WT and the D$_{211}$N version of Ty1 RT provides important clues to the question of why the mutant enzyme is blocked for *in vivo* transposition. The slower polymerization rate that we observed for single base addition could have a cumulative effect, making the possibility of complete synthesis of the 5.9 kb Ty1 double-stranded DNA much less likely. The decreased affinity of the mutant for divalent cations may affect occupancy of the metal binding sites at physiological concentrations of intracellular Mg$^{2+}$. Further, the pausing patterns that we observed for the mutant enzyme could represent absolute blocks at specific sequences along Ty1. While all of these effects probably contribute to reducing the viability of the mutant Ty1, the ability of second site substitutions in the presumably remote RNase H domain to restore transposition underscores the limits of our understanding of this phenomenon. The existence of these suppressors indicates that a delicate balance of polymerization, cleavage, and processivity must be maintained during transposition and that the D$_{211}$N mutation is well tolerated. Analysis of a recombinant form of the doubly mutant RT should reveal the basis for
the restoration of transposition competence, and efforts to express this form of the enzyme are currently underway.

**Acknowledgments**

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Special thanks to Rudra Dubey for technical help and suggestions, Ozcan Uzun for sharing earlier work on this topic and Natali Stano for advice and patient help with the quench-flow instrument.

**References**


**Figure legends**

**Fig. 1.** Purification of hexahistidine-tagged recombinant Ty1 RT by Ni²⁺-nitroloacetic acid-agarose affinity chromatography. 10% SDS-polyacrylamide gel shows purity of the 76.8 kDa fusion proteins at different steps of purification. Panel a, WT and Panel b, D₂₁₁N proteins. M depicts marker lane. In both panels, lane 1, crude extract; lane 2, wash with 25 mM imidazole; lane 3, wash with 80 mM imidazole; lane 4, purified protein eluted with 300 mM imidazole.

**Fig. 2.** Concentration dependence of the rate of single correct nucleotide incorporation by WT and D₂₁₁N Ty1 RT. Panels a and b show time courses of a single turnover at various dATP concentrations with the WT and D₂₁₁N enzyme respectively (□, 0.1 μM; ■, 0.5 μM; ▼, 1 μM; ▼, 2 μM; ◆, 5 μM; ●, 10 μM for WT in Panel a and ●, 0.1 μM; ◆, 0.4 μM; ▼, 0.8 μM; ▼, 3.3 μM; ■, 12.8 μM; □, 25.6 μM; ◆, 51.2 μM; ◆, 102.4 μM; ▲, 204.8 μM for mutant D₂₁₁N in Panel b). For WT, samples were taken at 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 3, 4, 6, 8, 10, 20, 30 seconds time points. For the mutant, samples were taken at 5 or 10 second intervals for the first minute and then every minute for the next 4 minutes. Panels c and d show pre-steady-state rate dependence of nucleotide incorporation on dATP concentration for WT and mutant respectively, based on the rates derived from Eqn. 1 (see supplementary information) in Panels a and b. Curves represent the best fits to Eqn. 2 (see supplementary information). The hyperbolic fit in Panel d was determined from two different sets of time courses.

**Fig. 3.** Extension patterns by WT and D₂₁₁N Ty1 RT under the influence of different...
divalent cations. Influence of divalent metals Mg$^{2+}$ and Mn$^{2+}$ were analyzed in vitro using 5' 32P-end labeled 14-mer/28-mer substrate, WT or mutant D211N Ty1 RT, and all four dNTPs (at 8.0 micromolar each). Lanes 1 and 2 represent reactions without divalent metal ions, 3 and 4 are reactions in the absence of any enzyme but in the presence of either divalent metal, lanes 5 - 8 are reactions in the presence of Mg$^{2+}$ and lanes 9 - 12 are those in the presence of Mn$^{2+}$. One hundred micromolar Mn$^{2+}$ was used after preliminary studies suggested that the WT enzyme was inhibited at higher concentrations.

**Fig. 4. WT and D211N mutant Ty1 RT polymerization as a function of metal ion concentration.** Polymerization reactions were carried out for 10 minutes in the presence of divalent ions ranging from 0 - 228 mM Mg$^{2+}$ (Panel a and b) and 0 - 49 mM Mn$^{2+}$ (Panel c and d) for both WT (Panel a and c) and D211N (Panel b and d).

**Fig. 5. Polymerization pausing patterns in DNA/DNA substrate.** Reactions were performed with the usual substrate in the presence of Mg$^{2+}$ (Panel a) or Mn$^{2+}$ (Panel b) with WT (Panel a lanes 1-6, Panel b lanes 1 and 2) or D211N (Panel a lanes 7 - 12, Panel b lanes 3 and 4). Reactions with or without unlabeled DNA traps are depicted as + and – respectively. Trap effectiveness controls are shown (Panel a lanes 13 and 14 and Panel b lanes 5 and 6).

**Fig. 6. Pausing patterns in DNA/DNA compared to those in DNA/RNA substrates.** Substrates were 5' 32P-end labeled 14-mer DNA primer/28-mer RNA or DNA template. Reactions conditions were the same as those in the previous experiment in the presence or the absence of trap (+ and – respectively). Reactions were carried out at 10 or 30 mM Mg$^{2+}$ or 2 mM Mn$^{2+}$ (WT; lanes 1 - 16 and D211N; lanes 17 - 32) for 10 minutes. Trap effectiveness controls (WT; lanes 1 - 4 and D211N; lanes 17 - 20) were done as before.

**Fig. 7. Processivity in long homopolymeric (oligo d(T)$_{14-18}$/poly dA or oligo d(T)$_{14-18}$/poly dA.**
rA) substrates. Analysis of processivity for WT (lanes 1 – 12) and D_{211}N (13 – 24) was done under a single round of primer extension using longer DNA or RNA templates in the presence of Mg^{2+} or Mn^{2+}. Klenow reactions were also carried out (lanes 25 to 32). Trap effectiveness control reactions for both the enzymes were performed in the presence of 10 mM Mg^{2+} (lanes 33 and 35 for WT and lanes 34 and 36 for D_{211}N). + or - depict with or without trap, respectively. Sizes are marked according to labeled marker.

Fig. 8. Dissociation of enzyme from its DNA substrate.

Ty1 RT (WT or D_{211}N) and labeled primer/template 14-mer/28-mer were mixed in a reaction mixture containing Mg^{2+} (10 mM final). Trap was then added. At various time points after addition of the trap, samples were withdrawn, and 200 micromolar dATP was added to initiate polymerization for 3 minutes. Graph shows the amounts of the extension products plotted against the time in minutes. Curves (●, WT Ty1 RT; ○, D_{211}N TY1RT; ▼, MMLV RT) show the best fit to the single exponential decay Eqn. 5. (see supplementary information).
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<th>$K_{d}Mn$ (mM)</th>
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<th>Hill constant Mn</th>
<th>$k_{off}$ (E•subs) (min$^{-1}$)</th>
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<td>7.8±0.4</td>
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<td>0.063±0.006</td>
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<td><strong>D$_{211}$N Ty1</strong></td>
<td>1.0±0.4</td>
<td>0.035±0.002</td>
<td>0.033±0.005</td>
<td>0.3916 ± 0.04</td>
<td>0.0073±0.003</td>
<td>1.92±0.36</td>
<td>2.3±0.18</td>
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<td>n. a.</td>
<td>n. a.</td>
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1. a.: not analyzed
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.

(a) Mg\textsuperscript{2+}(mM) and Trap controls

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<tr>
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(b) Mn\textsuperscript{2+}(mM) and Trap controls

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Fig. 6.
Fig. 7.
Supplementary Information for

Insights into the role of an active site aspartate in Ty1 reverse transcriptase polymerization

Reagents

Ampicillin, kanamycin, IPTG (isopropyl β-D-thio galactopyranoside), PMSF (phenyl methyl sulfonyl fluoride), lysozyme, imidazole, Nonidet P-40, activated calf thymus DNA were from Sigma. The nickel nitrilotriacetic acid (Ni-NTA) fast flow resin was from Qiagen. Denaturing acrylamide gel solutions were from National Diagnostics. Reagents and molecular weight size markers for the protein gels were from BioRad. Molecular size markers for DNA were from New England Biolabs. The dNTPs, polyr(A), poly r(C), poly d(A), oligo d(T)\textsubscript{14-18}, and oligo d(G)\textsubscript{14-18} were from Amersham Pharmacia Biosciences, Inc. Radiolabeled nucleotides were from Perkin Elmer Life Sciences. PAGE purified DNA oligonucleotides were from Integrated DNA Technologies, Inc. The 28-mer RNA template was from Dharmacon Technologies. T4 polynucleotide kinase and the Klenow fragment of *E. coli* DNA polymerase I were from New England Biolabs. Nucleotide removal kit was from Qiagen and P-30 columns were from BioRad. Recombinant Moloney murine leukemia virus RT was the gift of Dr. Millie Georgiadis (Indiana University).

Expression and Purification of Recombinant Ty1 RT

WT and mutant Ty1 RTs containing hexahistidine tags were expressed in *E. coli* strain M15[pREP4] (Qiagen) purified by Ni\textsuperscript{2+}-nitroloacetic acid-agarose (Qiagen) affinity chromatography as described (Wilhelm et al., 2000) with the following modifications.
Bacterial cells were grown up to 0.4 OD$_{600}$ in 2 liters of Luria Broth + ampicillin (100 μg per ml) + kanamycin (35 μg per ml), which was begun from an overnight culture at 37°C. Cultures were then incubated at 28°C, 200 r.p.m., and when the OD$_{600}$ approached 0.6, they were induced by addition of 0.5 mM IPTG and incubated for 8-12 hrs. Cells were harvested by centrifugation at 5000 r.p.m., in a GSA3 rotor (Sorvall) and resuspended in 2 ml RTA7/8 buffer (0.05 M sodium phosphate buffer, pH 7.8 with PMSF at final concentration of 1 mM) per gram of wet weight cells. Lysozyme was added to each suspension to a final concentration of 0.5 mg/ml and the suspension was incubated for 20-25 minutes on a rocker at 4°C. Sodium chloride was added to a final concentration of 300 mM and the suspension was sonicated (Sonifier 450, Branson) for 2 minutes with intermittent rest period at the 300 Watt setting. A cell free extract was prepared by centrifugation at 17,000 r.p.m. for 30 minutes using an SS34 rotor (Sorval) and the liquid phase was decanted. The cell free extract was passed through a 20 gauge syringe needle twice to reduce viscosity and then loaded onto a 1 ml Ni$^{2+}$-nitroloacetic acid-agarose column which had been pre-equilibrated with RTA7/8+300 mM NaCl+10% glycerol. The loaded samples were washed with 20 column volumes of RTA7/8+300 mM NaCl+25 mM imidazole+10% glycerol, followed by 20 column volumes of RTA6/0 (0.05 M sodium phosphate buffer, pH 6.0)+300 mM NaCl+25 mM imidazole+10% glycerol. Elution was carried out first with 3 column volumes of 80 mM imidazole in the RTA6/0+300 mM NaCl+10% glycerol buffer followed by 3 column volumes of 300 mM imidazole in the same buffer. Fractions containing RT activity as judged by the homopolymer substrate assay (described below), were pooled and dialyzed overnight against 150 mM NaCl, 50 mM Tris, 20% glycerol and 1 mM DTT buffer (pH 7.5) at 4°C.
The samples were then saved in the same buffer at –20°C with 50% glycerol. $5'\overset{\text{32P}}{-}$

**Labeling of oligonucleotides and DNA markers**

DNA oligonucleotides and 1kb and 100 bp marker ladders (New England Biolabs) were 5’ end-labeled using [$^{32}$P] ATP and T4 polynucleotide kinase. Unincorporated nucleotide was removed by nucleotide removal kit or by P-30 spun columns.

**Denaturing PAGE**

Reaction samples were quenched by mixing with loading buffer (95% formamide, 35 mM EDTA [pH 8], 0.1% bromphenol blue, 0.1% xylene cyanol). Samples were heated to 95°C for 5 min prior to loading 7 µl on a 1x TBE (0.089 M Tris-HCl, 0.089 M Boric acid and 0.002 M sodium EDTA buffered at pH 8.5), 7 M urea gels containing the appropriate percentage of polyacrylamide. Electrophoresis was performed in 1 x TBE buffer at ~1-1.2 watts/cm. Gels were visualized with a Storm 860 PhosphorImager and quantitated using the Image Quant 1.2 software (Molecular Dynamics).

**Primer Extension Assay with DNA Templates**

In the usual assay, a DNA/DNA template/primer was prepared by annealing a 28-mer plus-stand sequence from the polypurine tract region of Ty1 RT (5'-ATT ACA TTA TGG GTG GTA TGT TGG AAT A -3', where the polypurine tract is underlined) with a complementary 14-mer (5' T ATT CCA ACA TAC C 3') (template/primer ratio is 0.85/1), whose 5'-end is $^{32}$P-labeled, to generate the following model substrate:

\[
\begin{align*}
5' & \text{ATTACATTATGGGTGGTATGTTGGAATA} & 3' \\
3' & \text{CCATAACAACCTTAT*} & 5'
\end{align*}
\]

DNA substrate and enzyme were prepared in the extension buffer [17 mM Tris-HCl (pH 7.5), 17 mM NaCl, 1 mM dithiothreitol and 20% glycerol] and then pre-mixed with the
divalent cation (MgCl₂ or MnCl₂) and dNTPs in the extension buffer. The separate mixes were pre-warmed at 22°C for 10 minutes and were then combined in equal volumes to start the reactions at 22°C. Unless specified in the Materials and Methods section, an assay mixture contained 40 nM template-primer, ~18 nM of active WT or mutant D₂₁₁N Ty1 RT, with all four dNTPs (8 µM each and MgCl₂ or MnCl₂ at specified concentrations. Reactions were incubated at 22°C for 10 min., then terminated by the addition of loading buffer, denatured and separated by electrophoresis in 7 M urea - 17% polyacrylamide gels (or as specified time in Materials and Methods section). Using a phosphorimager, the amounts of the extension products (15-mer and greater) were determined relative to total labeled products seen (extended and un-extended 14-mer).

Eqn. 1:

\[
[P_{n+1}]_t = y_0 + [P_{n+1}]_{\text{max}} (1 - e^{(-k_t)_{\text{time}}})
\]

where \(k_t\) is the observed rate per second, \([P_{n+1}]_t\) is the amount of product at a given time, \(t\), \([P_{n+1}]_{\text{max}}\) is the maximum amount of product formed is in nM and \(y_0\) is the y intercept.

Eqn. 2:

\[
k = \frac{k_{\text{pol}} [\text{dATP}]}{K_d + [\text{dATP}]}
\]

Eqn. 3:

\[
[d\text{NTP} \cdot \text{Me}^{2+}]) = \frac{K_{d\text{ATP} + \text{Me}^{2+}} + [\text{dNTP}] + [\text{Me}^{2+}]}{2}\left(K_{d\text{ATP} + \text{Me}^{2+}} + [\text{dNTP}] + [\text{Me}^{2+}]ight) \cdot 4[\text{dNTP}]\cdot[\text{Me}^{2+}]
\]

where \([\text{Me}^{2+}]_{\text{total}}\) is the total metal ion concentration and free metal ion concentration \([\text{Me}^{2+}]_{\text{free}}\) was determined by subtracting the concentration of metal bound to dNTP from \([\text{Me}^{2+}]_{\text{total}}\).
Eqn. 4:

\[
\text{[product]} = y_o + \frac{[\text{product}]_{\text{max}} \cdot [\text{Me}^{2+}]^n_{\text{free}}}{(K_{d, so})^n + [\text{Me}^{2+}]^n_{\text{free}}}
\]

where \( n \) is the Hill constant, and product formed is in nM.

Eqn. 5:

\[
\text{[product]} = y_o + [\text{product}]_{\text{max}} \cdot e^{-k_{off}t}
\]

where product formed is Intensity of extended (>14-mer)/Intensity of total (≥14-mer), and time is in minutes.
Insights into the role of an active site aspartate in Ty1 reverse transcriptase polymerization
Manjula Pandey, Smita Patel and Abram Gabriel

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