Mutations in the Ribonuclease H Active Site of HIV−RT Reveal a Role for This Site in Stabilizing Enzyme−Primer−Template Binding

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ABSTRACT: The RNase H activity of HIV−RT is coordinated by a catalytic triad (E478, D443, D498) of acidic residues that bind divalent cations. We examined the effect of RNase H deficient $E^{478}\rightarrow Q$ and $D^{549}\rightarrow N$ mutations that do not alter polymerase activity on binding of enzyme to various nucleic acid substrates. Binding of the mutant and wild-type enzymes to various nucleic acid substrates was examined by determining dissociation rate constants (k_{off}) by titrating both Mg^{2+} and salt concentrations. In agreement with the unaltered polymerase activity of the mutant, the k_{off} values for the wild-type and mutant enzymes were essentially identical using DNA−DNA templates in the presence of 6 mM Mg^{2+}. However, with lower concentrations of Mg^{2+} and in the absence of Mg^{2+}, although both enzymes dissociated more rapidly, the mutant enzymes dissociated several-fold more slowly than the wild type. This was also observed on RNA−DNA templates. These results indicate that alterations in residues essential for Mg^{2+} binding have a pronounced positive effect on enzyme−template stability and that the negative residues in the RNase H region of the enzyme have a negative influence on binding in the absence of Mg^{2+}. In this regard RT is similar to other nucleic acid cleaving enzymes that show enhanced binding upon mutation of active site residues.

The process of human immunodeficiency virus (HIV)[1] replication requires the conversion of a single-stranded RNA to a double-stranded DNA provirus (*1*). HIV reverse transcriptase (RT) is the enzyme that is primarily responsible for this conversion. RT is a multifunctional enzyme that possesses both RNA- and DNA-dependent DNA polymerase activity and an RNase H activity (*2*).

During the process of viral replication, a variety of diverse nucleic acid structures are generated. RNA−RNA, RNA−DNA, and DNA−DNA hybrid structures are all present, as well as single-stranded RNA and DNA (*1*). To successfully replicate, RT must bind to these diverse substrates and catalyze both the polymerization activity and the RNase H activity to successfully proceed through the steps of replication. Although both activities are required, current therapeutic agents are directed only against the polymerase activity. Recent crystal structures of HIV−RT covalently bound to primer-templates have shown that numerous contacts between HIV−RT and primer-template occur throughout the enzyme (*3*). Determining how specific amino acid residues contribute to the interaction between HIV−RT and primer-template may play an important role in future drug design, especially in the development of nonnucleoside RT inhibitors.

All DNA polymerases, including reverse transcriptase, absolutely require a divalent metal cation, such as Mg^{2+} for catalytic activity (*4*). Mg^{2+} is the preferred cofactor for HIV−RT, and an in vitro concentration of 6−12 mM is optimal for reverse transcriptase activity (*5*). In addition to serving a catalytic function, previous work has indicated that Mg^{2+} serves to stabilize the binding of reverse transcriptase to DNA−DNA substrates (*6*). Crystal studies have shown that a single divalent cation is bound at the polymerase active site of HIV−RT [although this domain likely binds two divalent cations when performing catalysis (*7*)], while there still exists some debate as to whether 1 or 2 Mg^{2+} ions bind at the RNase H site (*8*−*12*). In the RNase H active site, the divalent cation(s) is (are) bound by the carboxyl groups of four acidic residues, Asp-443, Glu-478, Asp-498, and Asp-549 (*13*). The first three acidic residues are highly conserved in all bacterial and retroviral RNase H sequences (*14*).

A number of HIV−RT mutants have become available that possess a single amino acid change at the RNase H catalytic site [$E^{478}\rightarrow Q$ (*15*), $D^{443}\rightarrow N$ (*16*), $D^{498}\rightarrow N$ (*17*)]. Two of these mutants, $E^{478}\rightarrow Q$ and $D^{549}\rightarrow N$, eliminate the RNase H activity of RT, seemingly without affecting the polymerization or binding properties of the enzyme. The $E^{478}\rightarrow Q$ mutant has been used in several studies, including crystal studies detailing the interaction of HIV−RT with primer-template (*3*). In this paper, we have investigated the importance of this region in the binding of diverse nucleic

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[1] Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; rNTP, 2′-ribonucleoside 5′-triphosphate; dNTP, 2′-deoxynucleoside 5′-triphosphate; EDTA, ethylenediaminetetraacetate; AMP, adenosine 5′-monophosphate; BSA, bovine serum albumin; RNase H, ribonuclease H.

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acid substrates at a variety of Mg^{2+} concentrations. Experiments performed on *Bam*HI mutants indicate that changing residues from acidic to neutral at metal ion binding sites results in enhanced enzyme−DNA binding (*18*). Our results indicate that a large difference in binding affinity between mutant and wild-type enzymes is masked by high Mg^{2+} concentrations. Titrations done with varying amounts of Mg^{2+} illustrate that the wild-type enzyme dissociates very rapidly in comparison to the mutants, in the absence or presence of low concentrations of Mg^{2+}. The implications of these findings with respect to the role of the RNase H domain of RT in binding to nucleic acid substrates are discussed.

MATERIALS AND METHODS

Materials. Recombinant mutant ($E^{478}\rightarrow Q$) and wild-type HXB2 strain HIV−RT having the properties described (*15*) were used. Recombinant LAV strain HIV−RT having the properties described (*19*) was graciously provided to us by the Genetics Institute (Cambridge, MA). Aliquots of all HIV−RT were stored frozen at −70 °C, and a fresh aliquot was used for each experiment. T4 polynucleotide kinase was obtained from United States Biochemical Corp. T7 RNA, placental RNase inhibitor, poly(rA), rNTPs, dNTPs, and all restriction enzymes were obtained from Boehringer Mannheim; Sephadex G-50 columns and oligo$(dT)_{20}$ were obtained from Pharmacia. The DNA oligonucleotides used as primer and template strands (see Figure 1) were synthesized by Genosys Inc. (Houston, TX). All other chemicals were obtained from Fisher Scientific or Sigma Chemical Co. Radiolabeled compounds were from New England Nuclear.

Determination of Dissociation Rate Constants (k_{off}) by Nucleotide Incorporation. HIV−RT HXB-2 (wild type, $E^{478}\rightarrow Q$, and $D^{549}\rightarrow N$) (final concentration 20 nM) was preequilibrated with 5′-end-labeled primer-template (8 nM for both RNA−DNA and DNA−DNA substrates) for 10 min at 37 °C in 15 μL of 50 mM Tris-HCl (pH 8.0) 5 mM AMP, 0.1 mM EDTA, and 5−103 mM KCl (buffer A) depending on the desired ionic strength and the concentration of $MgCl_2$. A constant ionic strength was maintained in all reactions. To compensate for changes in ionic strength due to divalent cation changes, KCl was added to reactions with lower $MgCl_2$ concentrations. The ionic strength in solution is defined as half of the total sum of the concentration (c_i) of every ionic species (i) in the solution times the square of its charge (z_i); i.e., $I = 0.5\sum(c_i z_i^2)$. This equation indicates that $MgCl_2$ has 3-fold the ionic strength contribution of an equimolar concentration of KCl, so for every decrease in $MgCl_2$ in our reactions a commensurate 3-fold increase of KCl was introduced. AMP was added to inhibit any residual nuclease activities that may have been present in our enzyme preparations (*20*). A series of experiments were performed in the absence of AMP, and no difference was observed between reactions containing AMP and those that did not (data not shown). After preequilibration, 5 μg of poly(rA)−oligo$(dT)_{20}$ in 5 μL of buffer A was added to the reactions, and incubation was continued for varying amounts of time (depending on the KCl concentration and the template). The poly(rA)−oligo$(dT)_{20}$ was used as a trap to bind and sequester RT molecules that have dissociated from substrates (*20*). Poly(rA)−oligo$(dT)_{20}$ used in the reactions was prepared by mixing oligo$(dT)_{20}$ with poly(rA) at a 1:8 ratio (w/w), respectively, in 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. The mixture was incubated for 30 min at 37 °C and then slowly cooled to room temperature. Reactions were initiated by adding $MgCl_2$ and dNTPs in 5 μL of buffer A to give a final concentration of 6 mM $MgCl_2$ and 100 μM dNTPs. In some assays, Mg^{2+} (0.1−6 mM) was included during the preequilibration period. Reactions were run for 5 min at 37 °C and were terminated by addition of 25 μL of gel loading buffer [90% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol, 0.1% bromophenol blue]. Since the dissociation of the enzyme−nucleic acid substrate complexes is slow relative to the rate of polymerization (*21*), the amount of synthesis is proportional to the amount of bound RT at the initiation of reactions. The primer was extended by nine nucleotides. Samples were loaded onto a 10% polyacrylamide/7 M urea sequencing gel and subjected to electrophoresis as described (*22*). Quantification of extended and nonextended primer was accomplished by phosphorimager analysis of dried gels, using a GS-525 phosphorimager from Bio-Rad. The dissociation rates were determined by constructing a graph of incorporation, relative to the time zero sample, vs time. A nonlinear least-squares fit of the data to an equation for single-exponential decay [$f(x) = ae^{-bx}$, where $a = 1$ and b is the dissociation rate] using the Sigma Plot program (Jandel Corp.) was used to construct the graphs and determine the k_{off}.

Determination of the Rate of RNA Cleavage at Different Mg^{2+} Concentrations. Template-primer (100 nM) 5′-end-labeled on the RNA template strand was incubated in buffer A containing 0.1 μg/μL BSA and either 0.1, 0.5, 1.2, 2.2, 4.2, or 6 mM $MgCl_2$. Reactions were then initiated by the addition of enzyme to a final concentration of 2 nM. At varying time points 10 μL aliquots were quenched by the addition of an equal volume of gel loading buffer. Samples were electrophoresed and quantified as described above. The rate of enzyme cleavage was then determined by plotting the femtomoles of substrate cleaved per femtomole of enzyme vs time. The lines thus generated were used to calculate the rate of cleavage.

Measurement of RT Processivity under Varying Mg^{2+} Concentrations. The processivity of HIV−RT (wild type and $E^{478}\rightarrow Q$) was evaluated using conditions described above under Determination of Dissociation Rate Constants (k_{off}) by Nucleotide Incorporation Assays with the following changes: (1) all experiments were performed at 5 mM KCl; (2) the concentration of primer (10 nM) was twice that of the template (5 nM); (3) incubation after enzyme addition was 10 min in all cases; (4) reactions were initiated with dNTPs in a supplement containing the poly(rA)−oligo$(dT)_{20}$ trap. Samples were loaded onto a 6% polyacrylamide/7 M urea sequencing gel and subjected to electrophoresis as described (*22*).

Preparation of DNA Template for Processivity Assay. The DNA template was made using asymmetric PCR. Reactions contained 10 mM Tris-HCl (pH 8.85), 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 μg of plasmid pBSM13+ (Stratagene), 5 units of Pwo polymerase, 200 pM primer 1 (5′-CTTTATGCTTCCGGCTCGTA), and 2 pM primer 2 (5′-GAGTGCACCATATGCCATTCAGGCTACG-CAACTGTTGGGA). Conditions for PCR were 30 cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 min, followed by a final cycle of 70 °C for 5 min. Reactions

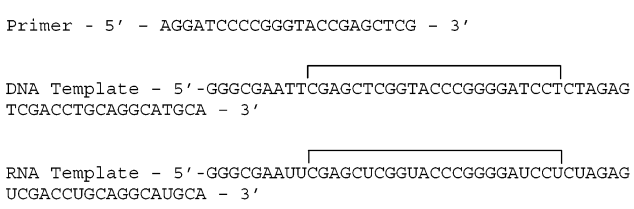

FIGURE 1: Substrates for binding experiments. Shown is the nucleotide sequence of the template and primer strands used in these experiments. The bracketed area above each template indicates the region where the primer strand is hybridized.

were electrophoresed on a 6% denaturing polyacrylamide gel, and the approximately 366 base single-stranded product was excised and recovered by passive elution.

Preparation of Hybrids. Hybrids were prepared by mixing the DNA or RNA template strand with a primer DNA that was 5′-^{32}P-labeled using T4 polynucleotide kinase. The hybrids were prepared by mixing primer-template at a 1:2 ratio in 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and varying KCl concentrations depending on the experiment. The mixtures were heated to 65 °C for 5 min and then slowly cooled to room temperature.

Preparation of RNA Template. Plasmid pBSM13+ (Stratagene) was cleaved with *Hin*dIII, and T7 RNA polymerase was used to prepare runoff transcripts 56 nucleotides in length. The manufacturer's recommended protocol was used for transcription reactions. Reactions were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. The RNAs were gel purified on denaturing polyacrylamide gels, located by ultraviolet shadowing, and recovered as previously described (*19*). The amount of recovered RNA was determined spectrophotometrically from the optical density.

Gel Electrophoresis. Denaturing polyacrylamide sequencing gels [19:1 acrylamide:bis(acrylamide) ratio], containing 7 M urea, were prepared and subjected to electrophoresis as described (*22*).

RESULTS

Design of the Nucleic Acid Substrate and Binding Assays Used To Measure HIV−RT Binding. Mutant and wild-type HIV−RT binding was measured on the nucleic acid substrates shown in Figure 1. The two substrates were of identical sequence except that the template strands were either RNA or DNA. These substrates were chosen because RT has been found to require a primer greater than 14 nucleotides in length for effective binding (*23*). In addition, enzymatic footprinting assays have shown that the enzyme protects template nucleotides +7 to −23 (*24*). Therefore, the 23 nucleotide primer corresponds to the area presumably associated with RT. HIV−RT binding to substrates was measured using various Mg^{2+} concentrations and a variety of ionic strength conditions. Dissociation rates (k_{off}) were determined as described in Materials and Methods.

In Figure 2, an autoradiogram of a typical k_{off} experiment is shown. Using 5′-end-labeled primer, extension was quantified at various time points. Those products that were extended run higher on the gel than unextended products. In the experiment shown in Figure 2, $E^{478}\rightarrow Q$ was equilibrated in the absence of Mg^{2+} with the RNA−DNA substrate for 10 min as described under Materials and Methods. A buffer containing the poly(rA)−oligo(dT)$_{20}$ trap was then added. The reactions were then initiated by the addition of 6 mM Mg^{2+} and 100 μM dNTPs at different times after trap addition. Values for k_{off} were obtained by fitting the data to an equation for single-exponential decay (see Materials and Methods).

Effect of Ionic Strength on Binding of Mutant and Wild-Type HIV−RT. It has been well established that the dissociation rate of HIV−RT from primer-templates increases as ionic strength increases. Table 1 lists a series of Mg^{2+} and ionic strength (KCl) concentrations used for these experiments. As expected at higher ionic strengths, dissociation of enzyme from the primer-template was substantially faster. In Mg^{2+} titration experiments, increasing the concentration of KCl as the level of $MgCl_2$ was decreased compensated for the ionic influence of the $MgCl_2$. Although the effect of divalent cation is quantitatively different at various salt concentrations, in all cases a large decrease in k_{off} was observed even at the lowest concentration of Mg^{2+} tested. This suggests that the changes result from association of Mg^{2+} with binding sites on the enzyme rather than any ionic or structural influence related to the template (*25*).

Data showed that Mg^{2+} stabilized the binding of both mutant and wild-type RTs to the DNA−DNA substrate under all tested conditions. However, the level of stabilization was highly dependent on the ionic strength conditions and the type of RT used. With lower ionic strengths (5 or 25 mM KCl), the addition of a small amount of Mg^{2+} (0.1 mM) enhanced binding to some extent for all of the tested enzymes. However, the wild-type enzymes showed significantly greater enhancement than the mutants. Even in the absence of Mg^{2+} the mutant RTs bound the substrate relatively tightly, dissociating about 12 times more slowly than the wild type (Table 1). At high Mg^{2+} concentrations (4.2−6 mM) binding of the mutant and wild-type enzymes was essentially identical. The results show that the mutations stabilize binding at low divalent cation concentrations and also in the absence of divalent cation, while this advantage is not observed under "optimum" conditions. This explains why others have found that these mutations have little or no effect on DNA polymerase activity (*15*).

Note that both the mutant and wild-type enzymes bound substrate significantly less stably in 80 mM KCl. In fact, dissociation rates could not be measured by the manual techniques employed for these assays if Mg^{2+} was not included. All of the enzymes were measurable when 6 mM Mg^{2+} was used, and all showed similar dissociation rates. With the $E\rightarrow^{478}Q$ mutant, binding was measurable with 0.5 mM Mg^{2+} or greater while the other enzymes required more Mg^{2+}. Clearly the enzymes bind considerably less tightly at higher salt concentrations. The fact that divalent cation had such a pronounced influence at high ionic strength may imply that relatively small changes in binding properties can manifest themselves as large changes in off-rates under weaker binding conditions.

Effect of the Divalent Cation on Binding of Mutant and Wild-Type Enzymes Using Low Ionic Strength Conditions. Titration of the binding of RT to the DNA−DNA substrate revealed a clear trend in the case of the wild-type enzyme. At 5 or 25 mM KCl concentrations, three binding modes were evident. As was noted above, at 5 mM KCl wild-type enzymes showed relatively poor binding without Mg^{2+}. This implies that the catalytic residues in the RNase H region of

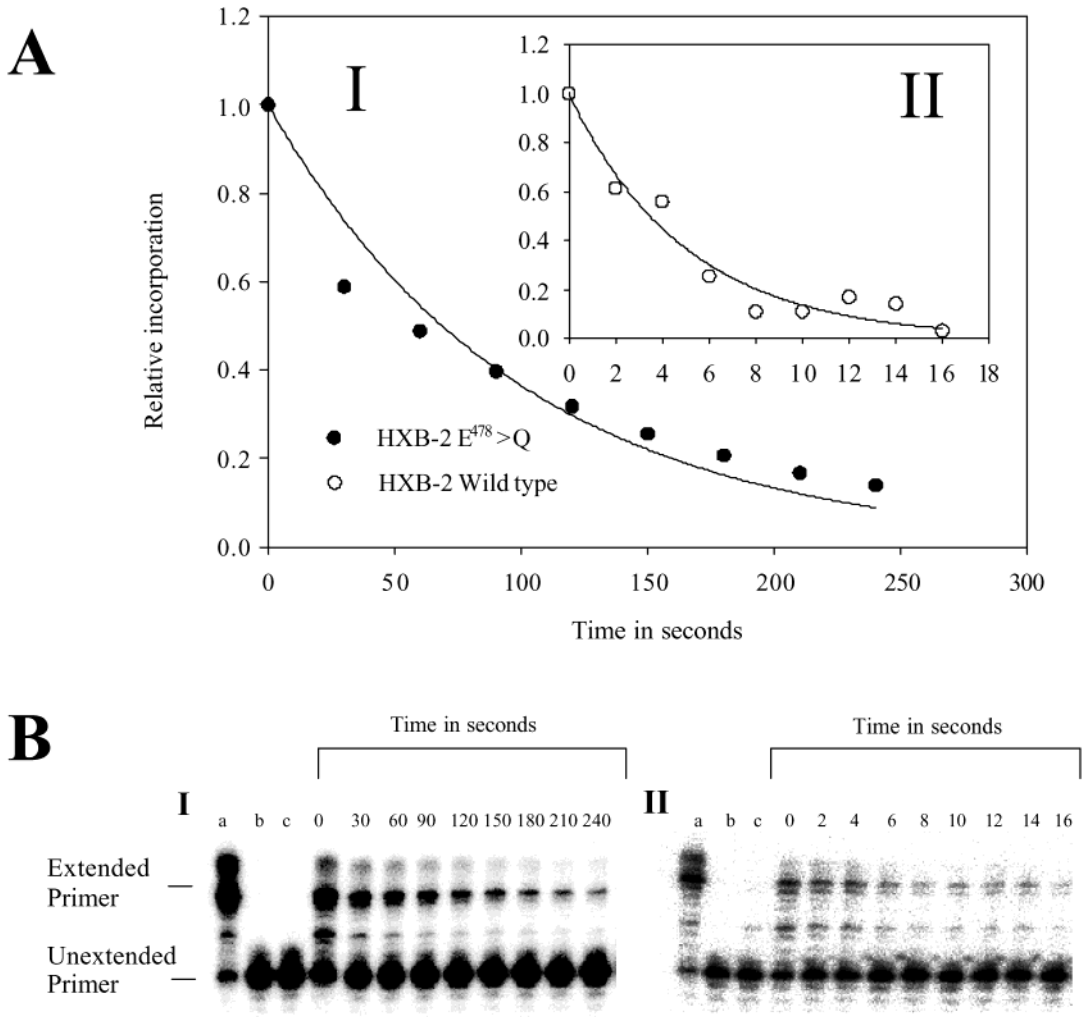

FIGURE 2: Determination of dissociation rates. Dissociation rates were determined by nucleotide incorporation as described under Materials and Methods. Panel A shows typical dissociation curves for DNA−DNA templates at 5 mM KCl in the absence of $MgCl_2$. Insert I represents a single-exponential fit of the data from a single experiment with HXB-2 E^{478}→Q. Insert II represents a single-exponential fit of the data from a single experiment with HXB-2 wild type. At least three independent experiments were used to determine the k_{off} value for this template. Relative incorporation represents the amount of primer extended in a particular lane relative to the amount of primer extended at the 0 point. Regression coefficients were 0.85 or greater. Panel B shows samples of gels used to determine the dissociation rate. Gels I (E^{487}→Q) and II (wild type): lanes a, control for extension in which the primer-template was mixed in the absence of trap; lanes b, control for enzyme in which primer-template was incubated in the absence of enzyme; lanes c, trap control in which primer-template was incubated with 5 μg of poly(rA)−oligo(dT) for 5 min before addition of enzyme. Time points for lanes are listed above the gel.

Table 1: Dissociation Rates (k_{off}) of DNA−DNA Substrates

	k_{off} (s^{-1})[a]						
	HXB-2 wt 5 mM KCl[b]	HXB-2 E^{478}→Q 5 mM KCl[b]	HXB-2 D^{549}→N 5 mM KCl[b]	HXB-2 wt 25 mM KCl[b]	HXB-2 wt 80 mM KCl[b]	HXB-2 E^{478}→Q 80 mM KCl[b]	HXB-2 D^{549}→N 80 mM KCl[b]
6.0 mM $MgCl_2$	0.007 ± 0.002	0.006 ± 0.0001	0.006 ± 0.0004	0.021 ± 0.003	0.26 ± 0.05	0.28 ± 0.08	0.15 ± 0.02
4.2 mM $MgCl_2$	0.007 ± 0.001	0.0037 ± 0.0006	0.0034 ± 0.0016	0.019 ± 0.001		0.19 ± 0.03	
2.2 mM $MgCl_2$	0.019 ± 0.004	0.0047 ± 0.0009	0.0041 ± 0.0003	0.016 ± 0.002		0.17 ± 0.03	
1.2 mM $MgCl_2$	0.016 ± 0.001	0.0035 ± 0.0003	0.0043 ± 0.0002	0.026 ± 0.001		0.17 ± 0.04	
0.8 mM $MgCl_2$	0.022 ± 0.003	0.0029 ± 0.0004	0.0034 ± 0.0002	0.055 ± 0.006		0.22 ± 0.03	
0.5 mM $MgCl_2$	0.012 ± 0.006	0.0076 ± 0.0005	0.0037 ± 0.0013	0.036 ± 0.0006		0.32 ± 0.03	
0.2 mM $MgCl_2$	0.012 ± 0.006	0.0051 ± 0.0004	0.0085 ± 0.0013	0.083 ± 0.001			
0.1 mM $MgCl_2$	0.02 ± 0.006	0.0059 ± 0.0018	0.0058 ± 0.0003	0.074 ± 0.001			
0 mM $MgCl_2$	0.15 ± 0.009	0.011 ± 0.0007	0.014 ± 0.003	0.12 ± 0.001			

[a] 5 mM KCl, 25 mM KCl, and 80 mM KCl refer to the amount of KCl added to 6 mM $MgCl_2$; at lower $MgCl_2$ concentrations more KCl was added to compensate for ionic strength. HXB-2, HXB-2 E^{478}→Q, and HXB-2 D^{549}→N refer to the strain from which the reverse transcriptase was derived. [b] Data collected from two to five independent experiments ± standard deviation.

the enzyme have a destabilizing effect on primer-template binding in the absence of Mg^{2+}. Addition of a small amount of Mg^{2+} enhanced binding about 10-fold, and binding was essentially constant between 0.1 and ~2.2 mM divalent cation. A further approximately 3-fold increase in binding affinity was observed at 4.2 mM Mg^{2+}. A similar three-mode trend was observed using 25 mM KCl with the wild-type enzyme although the inflection and fold levels of change

Table 2: Dissociation Rates (k_{off}) of RNA−DNA Substrates

	k_{off} (s^{-1})[a]					
	HXB-2 wt 5 mM KCl[b]	HXB-2 $E^{478}\rightarrow Q$ 5 mM KCl[b]	HXB-2 $D^{549}\rightarrow N$ 5 mM KCl[b]	HXB-2 wt 80 mM KCl[b]	HXB-2 $E^{478}\rightarrow Q$ 80 mM KCl[b]	HXB-2 $D^{549}\rightarrow N$ 80 mM KCl[b]
6.0 mM $MgCl_2$		0.0011 ± 0.0002	0.0023 ± 0.001		0.0025 ± 0.0006	0.0065 ± 0.0006
4.2 mM $MgCl_2$					0.0029 ± 0.0001	
2.2 mM $MgCl_2$					0.0038 ± 0.0002	
1.2 mM $MgCl_2$					0.0058 ± 0.0009	
0.8 mM $MgCl_2$					0.0052 ± 0.0014	
0.5 mM $MgCl_2$					0.0076 ± 0.0012	
0.2 mM $MgCl_2$					0.0085 ± 0.0006	
0.1 mM $MgCl_2$					0.0086 ± 0.0083	
0 mM $MgCl_2$	0.012 ± 0.01	0.0016 ± 0.0006	0.0023 ± 0.0002	0.43 ± 0.13	0.13 ± 0.02	0.27 ± 0.07

[a] 5 mM KCl and 80 mM KCl refer to the amount of KCl added to 6 mM $MgCl_2$; at lower $MgCl_2$ concentrations more KCl was added to compensate for ionic strength. HXB-2, HXB-2 $E^{478}\rightarrow Q$, and HXB-2 $D^{549}\rightarrow N$ refer to the strain from which the reverse transcriptase was derived. [b] Data collected from two to five independent experiments ± standard deviation.

were somewhat different. In contrast, at 5 mM KCl the mutant enzymes showed only about a 1.5−2-fold enhancement when comparing 0− 0.1 mM Mg^{2+}. Further small enhancements of binding occurred with increasing Mg^{2+} concentrations. It is interesting to note that on the DNA−DNA substrate at all ionic strengths with both wild-type and mutant enzymes, dissociation was slightly more rapid at 6 mM $MgCl_2$ than at 4.2 mM $MgCl_2$.

Binding of Mutant and Wild-Type Enzymes to RNA−DNA. On RNA−DNA templates at low ionic strength the difference in binding in the absence of Mg^{2+} for the mutants was less pronounced. In fact, the rate of dissociation at 6 and 0 mM Mg^{2+} fell within standard deviation of each other (see Table 2). At 80 mM KCl, however, the same trend seen on DNA−DNA templates can be observed, with even a small amount of $MgCl_2$ resulting in an approximately 25-fold increase in binding. Once again, the advantage of the mutants over the wild type in the absence of divalent cation was evident. The RNase H activity of the wild-type enzyme precludes testing in the presence of Mg^{2+} on RNA−DNA.

Effect of Histidine Purification Methods on Wild-Type and Mutant Binding Affinities. The HXB-2 enzymes (wild type, $E^{478}\rightarrow Q$, and $D^{549}\rightarrow N$) used in these experiments were recombinant enzymes that possessed an N-terminal histidine tag used to aid purification through a nickel column. To determine if the histidine tag had any effect on the dissociation of the enzymes from nucleic acid substrates, a wild-type enzyme lacking the histidine tag was tested [HIV−RT from LAV strain (*26*)]. Results indicated that wild-type LAV reverse transcriptase performed almost identically to wild-type HXB-2 reverse transcriptase (data not shown). This suggests that the histidine tag has little effect on overall binding.

Determination of RNA Cleavage Rates of Wild-Type HIV−RT under Varying Concentrations of Mg^{2+}. It appeared from the binding experiments on DNA−DNA that wild-type enzyme dissociated slower from primer-templates once a concentration of approximately 2.2−4.2 mM $MgCl_2$ was included. This may indicate that at these lower concentrations of Mg^{2+} the negative effect the RNase H catalytic residues have on overall primer-template binding is still present, albeit significantly reduced by the presence of some divalent cation. To determine whether the lower Mg^{2+} concentrations exhibited a similar effect on enzyme activity as on binding, we performed an RNase H cleavage assay at a variety of concentrations of $MgCl_2$ (0.1, 0.5, 1.2, 2.2, 4.2, and 6 mM). The data on cleavage rates under these various conditions are summarized in Table 3, and a representative experiment is shown in Figure 3. From the data gathered it is clear that the rate of cleavage is approximately 2 times greater at 6, 4.2, and 2.2 mM Mg^{2+} than at lower Mg^{2+} concentrations. The results indicate that only a very small amount of Mg^{2+} is necessary to occupy the catalytic site for RNase H activity and initiate cleavage but more is necessary for highly efficient cleavage. This implies that at least 1 Mg^{2+} ion occupies the RNase H active sites even at very low Mg^{2+} concentrations. Changing the divalent cation concentration below 2.2 mM (from 0.1 to 1.2 mM) had no effect on catalysis, which correlated with the k_{off} values. The enhancement in cleavage observed with the higher concentration of Mg^{2+} could be due to the observed increase in binding or a change in the rate of catalysis. The apparent increase in the rate of catalysis occurred at approximately 2 mM in these experiments, whereas in the off-rate experiment, a change in binding was not observed until the 4.2 mM point. However, it is important to note that the two assays cannot be directly correlated. The cleavage experiments represent a cumulative measurement of several variables including association rate, catalysis, and off-rate. Only the last parameter is measured in the binding experiments.

Table 3: Enzyme Cleavage Activity at Different Mg^{2+} Concentrations

$MgCl_2$ concn (mM)	fmol of template cleaved/ fmol of enzyme[a]
0.1	0.051 ± 0.009
0.5	0.047 ± 0.016
1.2	0.044 ± 0.026
2.2	0.092 ± 0.010
4.2	0.094 ± 0.008
6.0	0.099 ± 0.018

[a] Data collected from two to three independent experiments ± standard deivation.

Determination of Processivity of Wild-Type and $E^{478}\rightarrow Q$ Enzymes under Varying Concentrations of Mg^{2+}. The processivity of a polymerase is defined as the average number of nucleotides incorporated during a single binding event between the polymerase and substrate. In general, at a constant incorporation rate, a more stable interaction between the polymerase and primer-template leads to greater processivity. To determine if the observed binding affinities

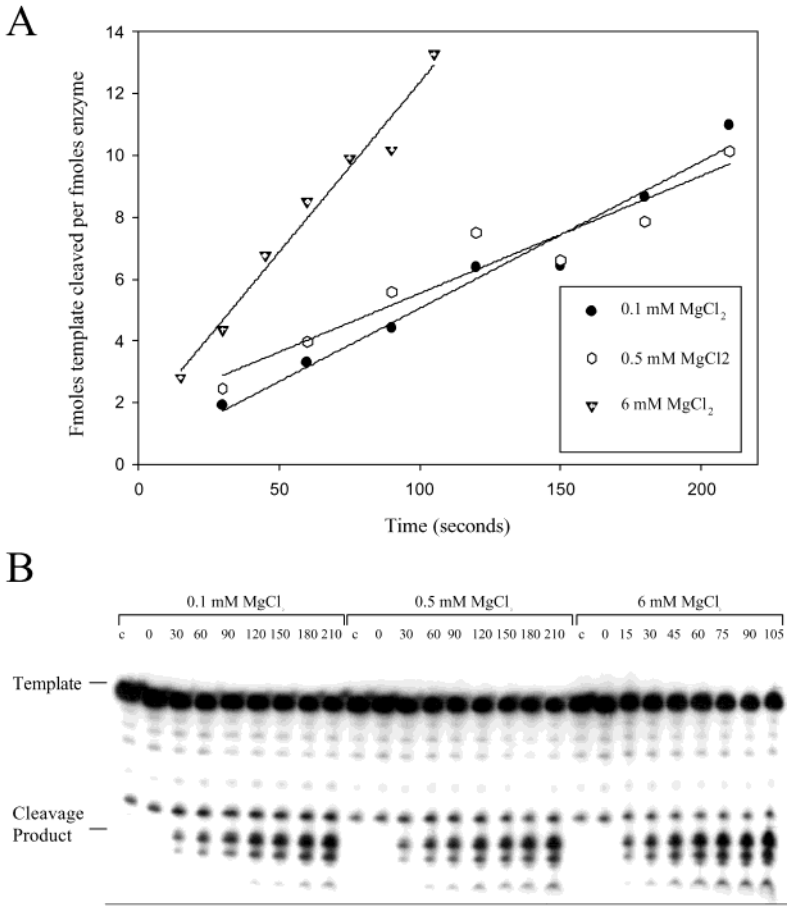

FIGURE 3: Determination of the rate of cleavage at various Mg^{2+} concentrations. Cleavage was determined as described under Materials and Methods. Panel A shows typical linear regressions for RNA−DNA templates at 5 mM KCl with varying concentrations of $MgCl_2$. The curve represents a fit of the data from the linear regression experiment with HXB-2 wild type at 0.1, 0.5, and 6 mM $MgCl_2$. Panel II shows a sample of the gel used to determine cleavage activity. Gel: lanes c, control for cleavage in which the primer-template was mixed in the absence of enzyme. Time points for lanes (in seconds) are listed above the gel.

correlate with a change in RT processivity, experiments with the wild-type and $E^{478}\rightarrow Q$ mutant enzymes were performed at 0.5, 2, and 6 mM $MgCl_2$ concentrations. If wild-type enzyme dissociates more rapidly than mutant enzymes at lower Mg^{2+} concentrations, then this should translate into a lower processivity for the wild type at reduced Mg^{2+} concentrations. A typical processivity experiment is summarized in Figure 4. The wild-type enzyme clearly has lower processivity than the $E^{478}\rightarrow Q$ mutant at lower Mg^{2+} concentrations (0.5 and 2 mM). This is evident from the relative reduction in large products in experiments with the wild-type enzyme. At 0.5 mM Mg^{2+}, very little product greater than 160 nucleotides was observed with the wild type. In contrast, the mutant enzyme showed a significantly greater proportion of large products. A small increase in the wild-type enzyme's processivity was observed with 2 mM Mg^{2+} while a more significant increase was noted at 6 mM. Mutant $E^{478}\rightarrow Q$ showed a clear increase in processivity at 2 mM in comparison to 0.5 mM while little increase was observed between 2 and 6 mM. At 6 mM Mg^{2+} the processivity of the $E^{478}\rightarrow Q$ and wild-type enzymes appeared similar. Experiments with the $D^{549}\rightarrow N$ mutant yielded results similar to those observed with $E^{478}\rightarrow Q$ (data not shown). This is consistent with the RNase H and dissociation rate data in implying that wild-type enzymes dissociate from primer-templates faster than mutant enzymes in the absence of Mg^{2+}.

DISCUSSION

We have examined the binding of mutant and wild-type HIV−RT to a duplex nucleic acid structure. Our results indicate that enzymes with loss of function mutations in the catalytic residues of the RNase H region bind significantly better to RNA−DNA and DNA−DNA templates than the wild type in the absence of Mg^{2+}. This binding advantage was maintained until relatively high concentrations of Mg^{2+} (about 4 mM) were present, at which point the mutant and wild-type enzymes bound with similar stability. The results suggest that the RNase H active site region plays an important role in primer-template binding and that coordination of divalent cations at this site stabilizes binding in the wild-type enzyme. Others have shown that conservative mutations in the polymerase region that inhibit catalytic activity still bind nucleic acid with similar efficiency in the presence of Mg^{2+} (*27*). In addition, RNase H deficient mutants with mutations in catalytic residues show processivity values similar to those of the wild type (*28*). This was also the case with the $E^{478}\rightarrow Q$ mutant used in these studies (*15*). These results and the results from this report indicate that, in the presence of Mg^{2+}, negative to neutral mutations in putative active site residues bind substrate comparably to wild type.

The more stable binding of the mutants without Mg^{2+} may be due to a charge effect, which reduces the repulsion of

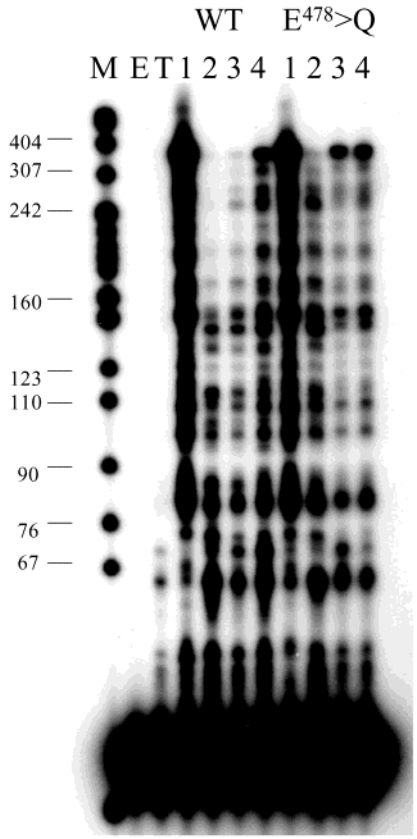

FIGURE 4: Determination of the processivity of wild type and E^{478}→Q at varying Mg^{2+} concentrations. Primer extension was carried out as described under Materials and Methods. Lanes: M, marker; E, enzyme control; T, trap control where the poly(rA)−oligo(dT) trap is incubated with wild-type enzyme and then added to the extension mix. Experimental lanes were performed for the wild-type and E^{478}→Q enzymes. Lanes: 1, extension control without trap; 2, 0.5 mM $MgCl_2$; 3, 2 mM $MgCl_2$; 4, 6 mM $MgCl_2$.

the active site residues for the negatively charged primer-template. In the presence of Mg^{2+} the catalytic residues of the wild-type enzyme would bind divalent cation and thus neutralize any negative charges that result in a difference in binding between the mutant and wild type. A similar effect was found with other nucleic acid binding proteins. *Bam*HI showed a 100−1000-fold increase in binding stability when mutants with negative to neutral changes in essential catalytic residues were bound to a nucleic acid substrate (*18*). *Mun*I endonuclease also showed a similar trend upon mutation of catalytic residues (*29*). Charged residues in the RNase H active site could directly weaken binding by repelling the nucleic acid substrate or may prevent the nucleic acid from obtaining a more stable binding conformation. This could occur if the substrate is forced to bind in a manner that alters its interactions with other regions of the enzyme. One point that argues against the observations being solely charge related was the dramatic difference in the off-rates for the mutant and wild type. The mutations, although eliminating only one of four negative charges in the region (see the introduction), caused a 10−15-fold decrease in off-rate. The fact that two separate mutations in the RNase H catalytic region resulted in a similar effect suggests that this is a general consequence of altering amino acids in this region. A second possibility is that the mutations stabilize binding through promoting a conformational change in the enzymes. For example, the mutants may assume a conformation closer to what occurs with the wild type in the presence of Mg^{2+}. Although the exact mechanism of stabilization is not evident from these experiments, it is clear that interactions between the catalytic residues of RNase H, divalent cation, and primer-template are important in stabilizing the binding of substrate to RT. It would be interesting to see if binding stability can be further strengthened using mutants with two or more changes in the RNase H catalytic site. This may be the case if the observed changes with the single mutants are based solely on a charge effect.

RNase H activity and processivity results lend further support to the importance of the RNase H region in template binding. At lower Mg^{2+} concentrations a decrease in RNase H activity, roughly corresponding to the decrease in binding affinity observed in binding experiments, was noted (Figure 3). Processivity results suggested that at 6 mM Mg^{2+} both mutant and wild-type enzymes have similar processivities, but at lower divalent cation concentrations enzymes with mutations in the RNase H region are considerably more processive than wild-type enzymes (Figure 4). The increased processivity of the mutants is consistent with the decreased off-rates observed with low divalent cation.

It is interesting to note that titration of wild-type RT with Mg^{2+} resulted in three somewhat distinct binding modes (Table 1). A monophasic progression would be expected for titration of a single binding site or of multiple binding sites with similar affinities for divalent cation. Structural data and previous biochemical analysis of HIV−RT suggest that the enzyme possesses two to three binding sites for divalent cation (*8*−*12*). Calorimetric assays indicated that these sites have different affinities for divalent cation (*12*). Thus the observed modal effect could have resulted from titration of binding sites with different affinities for Mg^{2+}.

In the current work the binding of wild-type RT to primer-template was highly dependent on Mg^{2+} concentration. This is to be expected, as Mg^{2+} is an essential cofactor in the enzymes' catalytic functions. Interestingly, it appears that the presence of Mg^{2+}, in addition to its catalytic function, serves to stabilize the binding of enzyme to the primer-template. As was noted above, previous work by Engler and Jen-Jacobson showed a very similar effect on the binding of *Bam*HI to template, where negative to neutral mutations in the essential catalytic residues greatly stabilized binding (*18*). These authors suggest that divalent cations, in addition to catalytic activity, serve to shield the template from the negative charges of the catalytic residues and that mutations in these residues effectively replicate this effect without restoring catalytic activity. This is especially interesting as HIV−RT requires divalent cation at both the polymerase and RNase H active sites. Results presented here imply that the RNase H active site plays an important role in primer-template binding as single negative to neutral mutations in this region have a pronounced effect on primer-template binding. The implication for HIV−RT that is supported by this work is that both RNase H and the polymerase active sites contribute significantly to primer-template binding. The idea that the combination of these two regions contributes far more to primer-template binding than one would expect for either the polymerase or the RNase H region alone is an intriguing concept. CheR, a signal transduction protein in *Escherichia coli*, contains two separate active sites connected via a "tether" region. Binding experiments have shown the

two binding sites create a synergistic effect; the combined protein binds significantly tighter to substrate than either individual region (*30*). Further experiments will be necessary to determine if this is also the case for HIV−RT. Information concerning the importance of the RNase active site of RT in binding substrate may be useful in future drug design, as disrupting interactions between enzyme and template within this region may have profound consequences in enzyme function.

Overall results show that the RNase H catalytic sites of HIV−RT that are involved in the coordination of divalent cations play an important role in primer-template binding. In the presence of Mg^{2+} the RNase H domain clearly stabilizes the association of RT with primer-template. It would be interesting to see if mutations in the polymerase active site effect binding in the absence of divalent cation in a similar manner.

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