Rate-limiting Pyrophosphate Release by HIV Reverse Transcriptase Improves Fidelity

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Previous measurements of the rates of polymerization and pyrophosphate release with DNA templates showed that pyrophosphate (PPi) dissociation was fast after nucleotide incorporation so that it did not contribute to enzyme specificity (kcat/Km). Here, kinetic parameters governing nucleotide incorporation and PPi release were determined using an RNA template. Compared with a DNA template of the same sequence, the rate of chemistry increased by up to 10-fold (250 versus 24 s⁻¹), whereas the rate of PPi release decreased to approximately 58 s⁻¹ so that PPi release became the rate-limiting step. During processive nucleotide incorporation, the first nucleotide (TTP) was incorporated at a fast rate (152 s⁻¹), whereas the rates of incorporation of remaining nucleotides (CGTCG) were much slower with an average rate of 24 s⁻¹, suggesting that sequential incorporation events were limited by the relatively slow PPi release step. The accompanying paper shows that slow PPi release allows polymerization and RNase H to occur at comparable rates. Although PPi release is the rate-determining step, it is not the specificity-determining step for correct incorporation based on our current estimates of the rate of reversal of the chemistry step (3 s⁻¹). In contrast, during misincorporation, PPi release became extremely slow, which we estimated to be ~0.002 s⁻¹. These studies establish the mechanistic basis for DNA polymerase fidelity during reverse transcription and provide a free energy profile. We correct previous underestimates of discrimination by including the slow PPi release step. Our current estimate of 2.4 × 10⁶ is >20-fold greater than estimated previously.

HIV-1 reverse transcriptase (HIVRT) is a DNA- and RNA-dependent DNA polymerase responsible for viral RNA replication. The larger p66 subunit contains both the polymerase and RNase H active sites, which are separated by ~60 Å. The polymerase domain copies the viral genome through repetitive cycles of nucleotide (dNTP) incorporation, comprising ground state nucleotide binding, enzyme conformational change, chemistry, and pyrophosphate (PPi) release steps (1–3). At the end of this cycle, HIVRT has to translocate from the pre- to the post-translocational state to empty the occupied nucleotide binding site (4, 5). Numerous structural and biochemical analyses have been performed to elucidate mechanisms of the first three steps of a nucleotide addition cycle, especially the dNTP binding-induced conformational change (6–8) and catalysis (9, 10) steps. Notably, recent studies have demonstrated that the nucleotide-induced conformation change governs the HIVRT specificity (1).

However, little is known about the mechanisms and kinetics governing PPi release and translocation. “Power-stroke” versus “Brownian ratchet” models for translocation have long been debated (11–13) since the concept now referred to as a Brownian ratchet was first proposed (14). Examination of the kinetics of processive synthesis by HIVRT using a DNA template reveals that each incorporation reaction proceeds at a rate approximately equal to the rate of chemistry observed in a single turnover (15, 16), suggesting that translocation must be faster than the rate of incorporation measured in a single turnover. Because the translocation step is kinetically “invisible” in rate measurements, a direct measurement of translocation by a polymerase has been sadly lacking until Malinen et al. (17) monitored RNA polymerase (RNAP) translocation along its DNA substrate using fluorescent base analogs and revealed fast translocation rates after incorporation of various nucleotides, with half-lives (t₁/₂) between 7 and 12 ms. Although atomic level details of PPi release by polymerases are still elusive (18, 19), it is generally thought to be fast (1, 2) with only few exceptions after mismatched or modified nucleotides are incorporated (20–22).

Our recent measurements of the rates of polymerization and pyrophosphate release by HIVRT in single turnover experiments with DNA templates showed that pyrophosphate (PPi) dissociation was fast after nucleotide incorporation so that it did not contribute to enzyme specificity (kcat/Km).³

In the current study kinetic parameters governing nucleotide incorporation and PPi release were determined using an RNA template to address questions as to how PPi release would affect the kinetics of sequential single nucleotide incorporation.
events during processive synthesis. Slow PP\textsubscript{i} release has been observed in previous studies of human mitochondrial DNA polymerase when incorporating modified nucleotides or mismatches with mutant enzymes (20–22), where it served to reduce the specificity constant. Herein, we examine how the slow PP\textsubscript{i} dissociation affects the nucleotide selectivity by HIVRT. In the accompanying paper we showed that the rate-limiting PP\textsubscript{i} release step during reverse transcription plays an important role in lowering the rate of processive synthesis to synchronize the polymerase and RNase H catalytic centers (24).

HIVRT has been an important target for antiretroviral therapy due to its critical roles in reverse transcription (25, 26). Drug resistance-associated mutations in the RT gene have been identified for all NRTIs (nucleoside/nucleotide analog reverse transcriptase inhibitors) approved by the United States Food and Drug Administration (27). The low fidelity of HIVRT has been proposed to make HIV-1 replication particularly error prone (28, 29), allowing the virus to develop resistance to antiretroviral drugs rapidly (30). Indeed, the fidelity of HIVRT has been reported to be 10-fold lower than the RTs from other sources (31–34). However, the true fidelity of HIVRT and its relative contribution to the high sequence variation of HIV are still debated. Fidelity measured in vitro using purified HIVRT proteins vary widely with different conditions (16, 29, 35–40), which, are typically around the 10\textsuperscript{-4} range (errors per nucleotide addition). However, in vivo studies have reported mutation frequencies ranging from 1.4 \times 10\textsuperscript{-5} to ~4 \times 10\textsuperscript{-5} per nucleotide per replication cycle (41–43), which are 5–20-fold lower than the error rate of HIVRT measured in vitro. Previous data from this laboratory have shown that slow PP\textsubscript{i} release provided a novel type of proofreading mechanism by allowing for the reversal of chemistry (20, 21), but this occurred only in rare cases. In this study the kinetics of correct and incorrect nucleotide incorporation were further examined to evaluate whether slow PP\textsubscript{i} release could bring the in vitro results in closer agreement with the in vivo data.

Results

**PP\textsubscript{i} Release Is Fast after DNA Polymerization with a DNA Template**—Fig. 1A shows the time dependence of nucleotide incorporation measured by rapid chemical quench flow methods after mixing a pre-formed enzyme-DNA duplex complex (175 nM MDCC-labeled WT_HIVRT and 75 nM d25/d45; Table 1) with various concentrations of TTP (0.5, 1, 2, 5, and 15 μM). B, kinetics of PP\textsubscript{i} release were measured by mixing a pre-formed enzyme-DNA duplex (200 nm MDCC-labeled WT_HIVRT and 300 nm d25/d45) with 1 mM TTP in the presence of reagents to measure PP\textsubscript{i} release as described under “Experimental Procedures.” C, global fitting of processive nucleotide incorporation data shown in panel E. Rates for the first, second, and third nucleotide incorporation were 23 \pm 2, 17 \pm 4, and 36 \pm 10 s\textsuperscript{-1}, respectively. D, global fitting of sequential processive nucleotide incorporation data shown in panel F. Rates for the first, second, and third nucleotide incorporation were 14 \pm 1, 26 \pm 3, and 30 \pm 5 s\textsuperscript{-1}, respectively. Red, 25-ntDNA primer; green, 26-nt product; blue, 27-nt product; yellow, 28-nt product; cyan, 29-nt product; magenta, 31-nt misincorporation product. E, processive nucleotide incorporation was measured by rapidly mixing a pre-incubated enzyme-d25/d45 complex (175 nM MDCC-labeled WT_HIVRT and 75 nM d25/d45) with 100 μM dATP, dCTP, and dGTP. Sequences for oligonucleotides used are given in Table 1. F, processive nucleotide incorporation was measured by rapidly mixing a pre-incubated enzyme-d25/d45 complex (175 nM MDCC-labeled WT_HIVRT and 75 nM d25/d45) with 100 μM dATP, dCTP, and dGTP. Positions of 25-nt primer and extension products with different lengths are marked. Smooth lines in each plot were derived by global fitting of each data set as described in the text.

**FIGURE 1.** PP\textsubscript{i} release was not a rate-limiting step during nucleotide(s) incorporation with DNA templates. A, rapid quench-flow assays were performed to measure the time dependence of product formation after mixing a pre-formed enzyme-DNA duplex complex (175 nm MDCC-labeled WT_HIVRT and 75 nm d25/d45) with various concentrations of TTP (0.5, 1, 2, 5, and 15 μM). B, kinetics of PP\textsubscript{i} release were measured by mixing a pre-formed enzyme-DNA duplex (200 nm MDCC-labeled WT_HIVRT and 300 nm d25/d45) with 1 mM TTP in the presence of reagents to measure PP\textsubscript{i} release as described under “Experimental Procedures.” C, global fitting of processive nucleotide incorporation data shown in panel E. Rates for the first, second, and third nucleotide incorporation were 23 \pm 2, 17 \pm 4, and 36 \pm 10 s\textsuperscript{-1}, respectively. D, global fitting of sequential processive nucleotide incorporation data shown in panel F. Rates for the first, second, and third nucleotide incorporation were 14 \pm 1, 26 \pm 3, and 30 \pm 5 s\textsuperscript{-1}, respectively. Red, 25-ntDNA primer; green, 26-nt product; blue, 27-nt product; yellow, 28-nt product; cyan, 29-nt product; magenta, 31-nt misincorporation product. E, processive nucleotide incorporation was measured by rapidly mixing a pre-incubated enzyme-d25/d45 complex (175 nM MDCC-labeled WT_HIVRT and 75 nM d25/d45) with 100 μM dATP, dCTP, and dGTP. Sequences for oligonucleotides used are given in Table 1. F, processive nucleotide incorporation was measured by rapidly mixing a pre-incubated enzyme-d25/d45 complex (175 nM MDCC-labeled WT_HIVRT and 75 nM d25/d45) with 100 μM dATP, dCTP, and dGTP. Positions of 25-nt primer and extension products with different lengths are marked. Smooth lines in each plot were derived by global fitting of each data set as described in the text.

sequent nucleotide incorporations (Fig. 1, C–F). The data were fit to a sequential incorporation model as shown in Scheme 2.

Rates for the first, second, and third nucleotide incorporations were 23 \pm 2, 17 \pm 4, and 36 \pm 10 s\textsuperscript{-1} for d25/d45\textsubscript{tsc} and 14 \pm 1, 26 \pm 3, and 30 \pm 5 s\textsuperscript{-1} for d25/d45\textsubscript{stc}. These results are consistent with the fast PP\textsubscript{i} release rate (> 500 s\textsuperscript{-1}) measured in the single turnover experiment because the rates are all comparable and appear to be limited by the rate of the chemistry step.

**PP\textsubscript{i} Release Becomes Rate-limiting for Processive Synthesis with an RNA Template**—The kinetics of TTP binding, incorporation, and subsequent PP\textsubscript{i} release were then examined using an RNA template. Five experiments (Fig. 2) were conducted and fit simultaneously to a single model as illustrated by Scheme 3. Fig. 2A shows the time dependence of nucleotide incorporation measured by rapid chemical-quench flow methods at several nucle-
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TABLE 1
DNA and RNA substrates for kinetic assays

The templating nucleotide(s) is shown in bold and underscored.

| d25r45                                      | d25, 5’-GGCTCGCAGCGCGTCCAACTCAAATCAGCA-3’ |
|---------------------------------------------|-------------------------------------------|
| r45, 3’-CGGACGGCCGGCGAGCGGCGAGCGGCGAGCGG-5’|                                            |

| d25f/r45                                   | d25f, 5’-GGCTCGCAGCGCGTCCAACTCAAATCAGCA-3’ |
|--------------------------------------------|-------------------------------------------|
| r45f, 3’-CGGACGGCCGGCGAGCGGCGAGCGGCGAGCG-5’|                                            |

| d25a/r45                                   | d25a, 5’-GGCTCGCAGCGCGTCCAACTCAAATCAGCA-3’ |
|--------------------------------------------|-------------------------------------------|
| r45a, 3’-CGGACGGCCGGCGAGCGGCGAGCGGCGAGCG-5’|                                            |

| d26f/r45                                   | d26, 5’-GGCTCGCAGCGCGTCCAACTCAAATCAGCA-3’ |
|--------------------------------------------|-------------------------------------------|
| r45f, 3’-CGGACGGCCGGCGAGCGGCGAGCGGCGAGCG-5’|                                            |

| d25f/d45                                   | d25, 5’-GGCTCGCAGCGCGTCCAACTCAAATCAGCA-3’ |
|--------------------------------------------|-------------------------------------------|
| d45, 3’-CGGACGGCCGGCGAGCGGCGAGCGGCGAGCG-5’ |                                            |

| d25f/d45                                   | d25, 5’-GGCTCGCAGCGCGTCCAACTCAAATCAGCA-3’ |
|--------------------------------------------|-------------------------------------------|
| d45r, 3’-CGGACGGCCGGCGAGCGGCGAGCGGCGAGCG-5’|                                            |

| d25f/d45                                   | d25, 5’-GGCTCGCAGCGCGTCCAACTCAAATCAGCA-3’ |
|--------------------------------------------|-------------------------------------------|
| d45, 3’-CGGACGGCCGGCGAGCGGCGAGCGGCGAGCG-5’ |                                            |

| d25f/d45                                   | d25, 5’-GGCTCGCAGCGCGTCCAACTCAAATCAGCA-3’ |
|--------------------------------------------|-------------------------------------------|
| d45r, 3’-CGGACGGCCGGCGAGCGGCGAGCGGCGAGCG-5’|                                            |

| d25f/d45                                   | d25, 5’-GGCTCGCAGCGCGTCCAACTCAAATCAGCA-3’ |
|--------------------------------------------|-------------------------------------------|
| d45, 3’-CGGACGGCCGGCGAGCGGCGAGCGGCGAGCG-5’ |                                            |

The templating nucleotide(s) is shown in bold and underscored.

**SCHEME 1.** The simplified model of TTP incorporation and PPᵢ release with a DNA template. In this model binding of nucleotide (N) to the enzyme-DNA duplex (ED₅₀N) is a rapid equilibrium process (K₆₅₀ = 3.5 μM) followed by the rate-limiting chemistry step (24 ± 1 s⁻¹). A lower limit (500 s⁻¹) is set for the fast PPᵢ (pyrophosphate) release, which is coincident with the chemistry. D₅₀ represents a 25-nt DNA primer annealed to a 45-nt DNA template.

| ED₅₀D₅₀ + N | ED₅₀D₅₀N | ED₅₀D₅₀PPi | ED₅₀D₅₀ + PPi |
|-------------|----------|------------|--------------|
| 3.5 μM      | 24 s⁻¹   | >500 s⁻¹   |              |

The rate constant governing the conformational change step, kₛ, was too fast to measure at 37 °C. Therefore, the experiments shown in Fig. 2 were repeated at lower temperatures to derive the maximum rate of nucleotide-induced conformational change (kₛ) because it was too fast to be measured at 37 °C. Traditionally, the extrapolation was achieved by Arrhenius plot. Here, we fit the temperature-dependent data globally to obtain the rate constants at 37 °C (Fig. 3). Briefly, a series of experiments covering a range of temperatures (5, 10, 15, 20, and 25 °C) was performed, then the family of curves were fit simultaneously to a single set of rate constants (Scheme 3) at the reference temperature (37 °C) and a corresponding set of activation energy terms to cover the range of temperatures. Global data fitting provided a maximum rate of kₛ = 3200 ± 90 s⁻¹ and ground state dissociation constant, 1/Kₛ = 520 ± 5 μM at 37 °C. The value of kₛ was then fixed in the fitting of all five experiments shown in Fig. 2 such that all remaining rate constants were derived from the global data fitting of data in Fig. 2 (Table 3).

The rate of TTP release from a pre-formed ternary complex was measured by mixing the closed complex with unlabeled trap (Fig. 2C). 3’-Dideoxy-terminated primer was used in this assay to block the chemistry step. Because the ground state binding of TTP is a fast equilibrium process, re-opening of the ternary complex is the rate-limiting step for TTP dissociation and was defined by the assay. This yielded a k₋₋ value of 3 ± 0.1 s⁻¹, which was well constrained in the global fitting according to confidence contour analysis (not shown). The rate of chemistry was 250 ± 25 s⁻¹, which was 10-fold faster than the rate obtained with a DNA template (24 ± 0.1 s⁻¹, Fig. 2A and Scheme 1), consistent with prior single turnover measurements (44, 45). The rate of PPᵢ release was examined using a coupled assay previously developed in our laboratory (46) in which PPᵢ released from the reaction was hydrolyzed by the pyrophosphatase, yielding inorganic phosphate, which then bound to the fluorescently labeled *Escherichia coli* phosphate-binding protein (PPB) to give the fluorescence signal (Fig. 2D). The kinetics of PPᵢ hydrolysis and PPᵢ binding to MDCC-PBP were calibrated as previously described.3 Interestingly, the fitting afforded a PPᵢ release rate of 58 ± 2 s⁻¹ that was ~4-fold slower than the rate of chemistry. Therefore, product release became the rate-limiting step (Fig. 2D). Consequently, due to the slow PPᵢ release step, the rate constant governing the reversal of chemistry (k₋₋) was able to be accurately determined as 3.5 ± 0.5 s⁻¹ by measuring the kinetics of pyrophosphorolysis (Fig. 2E). The experiment also defined a second order rate constant for PPᵢ rebinding of 1.2 ± 0.4 μM⁻¹s⁻¹. This data set (Table 3) provided the first complete free energy profile for DNA polymerization catalyzed by HIVRT described below (see Fig. 8).

**Kinetics of Processive Synthesis**—Processive nucleotide incorporation was examined to further explore the possible consequences of the rate-limiting PPᵢ release step (Fig. 4). Incorpora-
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TABLE 2
Specificity constants and rates of PPi release of TTP incorporation by various RTs in complex with different substrates

| Enzyme                  | Substrates | $k_{pol}$ | $k_{d,app}$ | $k_{pol}/k_{d,app}$ | PP, release |
|-------------------------|------------|-----------|-------------|---------------------|-------------|
| MDCC-WT_HIVRT           | d25/g45    | 24 ± 0.1  | 3.5 ± 0.4   | 6.9 ± 0.8           | >500        |
| MDCC-WT_HIVRT           | d25/r45    | 201 ± 6   | 37 ± 3      | 5.4 ± 0.5           | 58 ± 0.4    |
| Unlabeled WT_HIVRT      | d25/r45    | 192 ± 8   | 24 ± 1      | 8 ± 0.5             | 35 ± 1      |
| Unlabeled WT_HIVRT      | d25pC/r45pG | 150 ± 10  | 34 ± 4      | 4.4 ± 0.6           | 28 ± 1      |

SCHEME 2. Sequential nucleotide incorporation model. Processive nucleotide incorporation data were fit to the model including multiple incorporation events (i.e. one-step incorporation of each nucleotide occurs repeatedly (from N1 to N3)), including dissociation of intermediate products (e.g. $D_{25pC}D_{45}$). $X_{25pC}D_{45}$ is the non-productive complex that can slowly switch to the polymerization-competent mode ($ED_{25pC}D_{45}$) (24). The same model was used to fit data involving more incorporation steps (Fig. 4 and 6) by introducing the corresponding number of incorporation events.

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ED₂₅R₄₅ + N → ED₂₅R₄₅N

SCHEME 3. Two-step nucleotide binding-induced-fit mechanism. D₂₅R₄₅ represents a 25-nt DNA primer annealed to a 45-nt RNA template. ED₂₅R₄₅N stands for the nucleotide-induced fingers closure.

TABLE 3
Kinetic parameters governing TTP binding and incorporation by MDCC labeled WT_HIVRT in complex with a DNA/RNA hybrid

| Parameter | Value  
|-----------|--------|
| 1/K₁ | 520 ± 5 |
| K₂ | 3200 ± 90 |
| K₃ | 250 ± 25 |
| K₄ | 3.5 ± 0.5 |
| K₅ | 58 ± 2 |
| K₆ | 1.2 ± 0.4 |
| K₇ | 0.5 ± 0.02 |
| K₈ | 201 ± 6 |
| K₉ | 8.3 ± 3 |
| K₁₀ | 54.6 ± 0.5 |
| K₁₁ | 46 ± 2 |
| K₁₂ | 5.7 ± 0.2 |

For comparison to the global fitting, these values were obtained from conventional data fitting with a hyperbolic function to fit the TTP concentration dependence of incorporation.

Incorrect Nucleotide Incorporation Also Revealed Rate-limiting PPᵢ Release—Rapid quench flow assays were performed to measure the kinetics of dGTP:PrA misincorporation by unlabeled WT_HIVRT in complex with d25/r45 (Fig. 7A). To further explore the slow reactions, the same experiments were repeated for extended timescales, either 5 (Fig. 7B) or 30 min (Fig. 7C). Our previous data have shown that the kinetics of mismatch nucleotide binding were complex, and most of the fluorescent signal disappeared within the 1-ms dead time of the stopped-flow instrument due to the fast opening rate after binding a mismatch (8). Consequently, only quench flow assays were performed, and a simplified mechanism involving one-step substrate binding was applied to describe the kinetics of dGTP misincorporation (Scheme 4). The kinetic parameters resulting from global analysis of kinetic data are summarized in Table 4. Data shown in Fig. 7A defined the rate of misincorporation or kₐp (approaches kₓ) as 0.6 ± 0.03 s⁻¹ and the apparent nucleotide dissociation constant or Kₐₚ of 11 ± 2 mM. However, a concentration dependence of the amplitude of dGTP incorporation was observed, implying that the chemistry step was reversibly linked to nucleotide binding (k₋₋ = 0.07 ± 0.01 s⁻¹) and implying the existence of a slow, rate-limiting step after chemistry (20, 21). Interestingly, the extension reaction...
revealed an additional slower phase when dGTP misincorporation was monitored for 5 min, where the slow phase was likely to correspond to the rate-limiting PP\textsubscript{i} release (20). Accordingly, the slow phase defined the rate of PP\textsubscript{i} release to be 0.003 ± 0.001 s\textsuperscript{-1}, which was 200-fold slower than the catalytic step and was probably limited by the reopening of RT after primer extension. It is likely that the enzyme remains in a closed conformation after the chemistry step, in which residues would still be in an alignment to perform catalysis (20). This hypothesis is consistent with the fact that the amplitude of the fast phase is dependent on dGTP concentration. Once the enzyme reopens and releases PP\textsubscript{i}, the reaction would precede to a common end point as PP\textsubscript{i} release is essentially irreversible due to the relatively low concentration of PP\textsubscript{i} formed during the course of the reaction (20). To test this, kinetics of misincorporation were monitored for 30 min (Fig. 7C). To our surprise, the dependence of reaction end point on nucleotide concentration was still observed, which fit with a PP\textsubscript{i} rebinding rate of <0.02 μM\textsuperscript{-1}s\textsuperscript{-1}. However, any number smaller than this rate could generate an acceptable fit. Data in the three panels in Fig. 7 were fit globally to derive rate constants summarized in Table 4.

Rate-determining Versus Specificity-determining Step—In this study the kinetic pathways of correct and incorrect nucleotide incorporation were identified (Schemes 3 and 4). Kinetic parameters governing nucleotide binding, incorporation, and concomitant PP\textsubscript{i} release are listed in Tables 3 and 4. The data showed that PP\textsubscript{i} release was the rate-determining step during TTP incorporation when an RNA template was used. Previous studies of nucleotide selectivity by HIVRT and T7 DNA polymerase identified an important kinetic partitioning between chemistry (k\textsubscript{c}) and the reverse of the conformational change (k\textsubscript{r}) that allows simplifications to be made when deriving the specificity constant (1, 47). Notably, when k\textsubscript{r} is much greater than k\textsubscript{c}−1, the specificity constant (k\textsubscript{cat}/K\textsubscript{m}) can be simplified to K\textsubscript{c}/k\textsubscript{r} because the closed ternary complex is committed to catalysis. In this case, the kinetic partitioning of the FD\textsubscript{25}R\textsubscript{45} N state favors chemistry over nucleotide release (k\textsubscript{r} ≥ k\textsubscript{c}). However, when chemistry proceeds, a new kinetic partitioning between PP\textsubscript{i} release (k\textsubscript{r}) and the reverse of chemistry (k\textsubscript{c}) also becomes important. Because k\textsubscript{r} ≥ k\textsubscript{c}, the specificity constant governing the incorporation of a normal nucleotide (TTP) is not affected by the slow PP\textsubscript{i} release; rather, it is solely determined by the net rate of binding (K\textsubscript{c}/k\textsubscript{r}). Again, the data demonstrate that nucleotide selectivity by HIVRT is determined by the first largely irreversible step, which is the substrate-induced isomerization. The free-energy profile for TTP binding and incorporation illustrates this analysis. In Fig. 8A, the nucleotide-induced conformational change (ED\textsubscript{25}R\textsubscript{45} N to FD\textsubscript{25}R\textsubscript{45} N) represents the highest energy barrier relative to the unbound state (ED\textsubscript{25}R\textsubscript{45}) and, therefore, determines the enzyme specificity constant (k\textsubscript{cat}/K\textsubscript{m} = K\textsubscript{c}/k\textsubscript{r}). Although PP\textsubscript{i} release reflects the highest absolute barrier (relative to the local minimum) and, therefore, represents the rate-limiting step...
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**FIGURE 6.** Processive nucleotide incorporation by unlabeled WT_HIVRT also supported slow PPi release. A, global fitting of processive nucleotide incorporation data shown in panel C. Rates for sequential nucleotide incorporation were 98 ± 6, 22 ± 2, 32 ± 4, 33 ± 5, 21 ± 3, and 27 ± 6 s⁻¹. Red, 25-nt DNA primer; light green, 26-nt product; blue, 27-nt; yellow, 28-nt; cyan, 29-nt; magenta, 30-nt; green, 31-nt; purple, 35-nt misincorporation product. B, global fitting of processive nucleotide incorporation data shown in panel D. Rates for sequential nucleotide incorporation were 92 ± 11, 44 ± 7, 34 ± 6, 23 ± 4, and 29 ± 7 s⁻¹. Red, 26-nt DNA primer; light green, 27-nt product; blue, 28-nt; yellow, 29-nt; cyan, 30-nt; magenta, 31-nt; green, 35-nt misincorporation product. Processive nucleotide incorporation was measured by rapidly mixing a preincubated enzyme-DNA/RNA complex (175 nM unlabeled WT_HIVRT and 75 nM d25pC/r45pG (c) or d26pC/r45pG (D)) with 100 μM TTP, dCTP, and dGTP. Sequences of substrates are shown on the top of each panel. Positions of primers and extension products with different lengths are marked. Timescales are shown on the bottom of each panel. The labels in panels A and B show the sequence of nucleotides incorporated.

**FIGURE 7.** Global fitting of dGTP:rA misincorporation by unlabeled WT_HIVRT. A, kinetics of misincorporation were measured by rapid quench flow assays after mixing a preformed enzyme-DNA/RNA complex (175 nM unlabeled WT_HIVRT and 75 nM d25pC/r45pG) with various concentrations of dGTP (0.2, 0.5, 1, 2, and 4 μM). Reaction were stopped at various time points within 10 s by the addition of 0.5 mM EDTA. B, the same experiments were repeated by hand quench, and data were collected up to 5 min. C, the same experiments were repeated by hand quench, and data were collected within 30 min. The three experiments performed with different timescales were fit simultaneously according to the one-step binding model shown in Scheme 4, as shown by the smooth lines in each panel. Rate constants derived from the global analysis were listed in Table 4.

**TABLE 4.** Kinetic parameters governing dGTP binding and misincorporation by unlabeled WT_HIVRT.

| Parameter | Value |
|-----------|-------|
| $k_{d_{app}}$ | $k_{cat}$ |
| $k_{app}$ | $k_{cat}$ |
| $k_{-2}$ | $k_{-3}$ |
| $k_{-3}$ | $k_{-3}$ |

| $K_{d_{app}}$ | $K_{cat}$ |
|---------------|------------|
| $K_{d_{app}}$ | $K_{cat}$ |
| $K_{cat}$ | $K_{cat}$ |
| $D^*$ | $D^*$ |

$^{*}k_{cat}$ is not well determined by the data.
$^{*}k_{cat}/K_{cat} = k_{cat}(k_{cat} + k_{cat} + k_{cat})$. The difference between the specificity constants obtained by including or overlooking the slow PPi release step was defined by $D = (k_{pol}/K_{d_{app}})/(k_{cat}/K_{cat})$.

(k₃) approximates $k_{cat}$, it does not contribute to enzyme specificity, which is further supported by the fact that $k_{pol}/K_{d_{app}} = k_{cat}/K_{cat}$ (Table 3).

The scenario changes during noncognate nucleotide incorporation, in which the product release rate ($k_3$) was shown to reduce the specificity constant, $k_{cat}/K_{cat}$. The slow PPi release rate allows for the reversal of chemistry ($k_{-2}$), which effectively reduced $k_{cat}/K_{cat}$ by 24-fold according to the constants defined for dGTP incorporation (Table 4). The data demonstrated that when $k_{-2} \gg k_3$ (note different numbering for misincorporation, Scheme 4), an equilibrium was established prior to product release, which results in a simplified derivation of specificity corresponding to product of the equilibrium constants and the rate of product release ($K_{cat}k_3$) (47). This is further illustrated in the free-energy profile for dGTP incorporation (Fig. 8B) where the $k_{pol}$ product release reflected the highest barrier relative to the unbound state and the local minimum. Therefore, slow product release represents the rate- and specificity-determining step during noncognate nucleotide incorporation.

**Discussion**

One of the most important results of this report is the identification of rate-limiting PPi release during DNA polymerization catalyzed by HIVRT with an RNA template. PPi release has usually been assumed to be fast, so chemistry represents the only rate-limiting step for primer extension, and direct measurement has supported that model for DNA-dependent DNA polymerization. As shown in Fig. 1B, the rate of PPi release after incorporation of TTP using a DNA template must be quite fast (~500 s⁻¹) as the observed fluorescence signal corresponding to PPi, is coincident with the observed chemistry. Examination of the kinetics of processive synthesis using a DNA template reveals that each incorporation reaction proceeds at a rate approximately equal to the rate of chemistry observed in a single turnover (15, 16). Therefore, there is no significant lag after the incorporation of one nucleotide and preceding the binding of the next. The inability to see a delay in binding of the next nucleotide after a single incorporation event (Fig. 1, C–F) also supports that translocation must be faster than the rate of incorporation measured in a single turnover with a DNA template.
In contrast, the data revealed that PP$_i$ product release represents the rate-limiting step when an RNA template is used (Fig. 2). This conclusion is further supported by the observation of significantly delayed incorporation of the next nucleotide after the first turnover (Fig. 4). Either rate-limiting PP$_i$ release or translocation could account for the kinetic phenomenon. The mechanistic basis for translocation is still elusive although several models have been proposed. Based on the structural analysis of T7 RNA polymerase, a power stroke model was proposed, where dissociation of PP$_i$ drives translocation by generating the power stroke conformational change (active site opening) (11). However, other studies with DNA or RNA polymerases argue directly against power-strokes models that are tightly coupled to PP$_i$ release or dNTP binding (12, 13). Rather, data from these studies are consistent with the Brownian ratchet mechanism where the polymerase oscillates between the pre- and post-translocation states, and the binding of next correct nucleotide stabilizes the post-translocational stage (14). In either model, a rate-limiting translocation seems to be infeasible, although the possibility cannot be fully excluded. Monitoring of RNAP translocation along its DNA substrate using fluorescent base analogs revealed fast translocation rates after the incorporation of various nucleotides, with half-lives ($t_{1/2}$) between 7 and 12 ms (17).

Direct measurements of PP$_i$ release clearly demonstrate that product release is indeed rate-limiting when an RNA strand is the template. Although slow PP$_i$ release has been reported during the incorporation of modified or mismatched nucleotides by the human mitochondrial DNA polymerase by the human mitochondrial DNA polymerase (20–22), this is the first instance where rate-limiting PP$_i$ release is observed using cognate nucleotides. Slow intrinsic off-rate of PP$_i$ was also measured for the E. coli RNAP elongation complex in the absence of next cognate nucleotide; the addition of nucleotides during processive transcription may increase the rate to physiologically relevant value (48). However, dissociation rates of PP$_i$ by HIV-1 RT measured in this study were still slow even under the processive nucleotide incorporation conditions, implying a distinct mechanism.

To date very little is known about the atomic-level mechanism of PP$_i$ release, although sparse models have been proposed to address this question. Based on molecular dynamics (MD) simulations, a “hopping” model for PP$_i$ release was proposed, where a few positively charged residues along the exit channel form the hopping sites and facilitate the process of PP$_i$ release (18). Another MD simulation study of a Y-family DNA polymerase (human Pol η) suggested that a highly flexible and conserved arginine residue (Arg-61) acts concertedly with a third transient metal ion that forms an “exit shuttle” for the leaving PP$_i$ group (19). The actual PP$_i$ release should be a fast process if the channel connecting the internal active site to the external solvent is large enough for a PP$_i$ molecule to freely diffuse (49). However, a PP$_i$ molecule may not be able to diffuse through the exiting tunnel if the incorporating nucleotide is deeply buried and has only limited access to the protein surface (50, 51). In this case, the PP$_i$ molecule needs to wait until the fingers domain reopens before it can exit the active site.

As to HIV-1 RT, the β3-β4 loop of its fingers domain closes down on the triphosphate portion of the incoming dNTP (6, 8), which probably constrains the free diffusion of PP$_i$ after the phosphoryl transfer step. Consequently, it is reasonable to suppose that the relatively slow product release is limited by the isomerization of HIVRT to the open state. This hypothesis was supported by the fact that reopening of RT measured by stopped-flow fluorescence assays (Fig. 2B) is coincident with PP$_i$ release measured by our coupled stopped-flow assay (Fig. 2D). That is, the data clearly show faster chemistry and a slower rate of enzyme reopening, which is coincident with PP$_i$ release. Although these data do not demonstrate the order of events, it is reasonable to propose that enzyme opening precedes and, therefore, limits the rate of PP$_i$ release. Alternatively, the power stroke model for translocation implied that the PP$_i$ release step precedes the active site reopening (11), which, however, were not compatible with results from other studies that supported a Brownian ratchet model (12, 13). Our previous MD simulations showed that HIVRT opens rapidly in the absence of PP$_i$ (8, 13).

**FIGURE 8.** Free-energy profiles for TTP (A) and dGTP (B) incorporation. A, the free energy was calculated as $\Delta G = RT\ln(k/T) - \ln(k_{diss})$ kcal/mol using rate constants from Table 3. The constant $k$ is the Boltzmann constant, $T$ is 310 K, $h$ is Planck’s constant, and $k_{diss}$ is the first-order rate constant. For second order reactions, a pseudo-first order rate constant was calculated using nominal physiological concentrations of nucleotide and PP$_i$ 100 and 150 μM, respectively. B, the free-energy profile for dGTP misincorporation was generated using rate constants from Table 4. Misincorporation reveals only a single binding step as described previously because the initial binding is weak and the conformational change is fast but unfavorable (8).
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we are currently extending these studies to examine the rate of enzyme opening with bound PPi.

An overlay of RT structures in complex with a DNA duplex (3V4L.pdb; Ref. 52) or a DNA/RNA hybrid (4PQUpdb; Ref. 53) may be expected to shed some light on the structural basis of rate-limiting PPi release. However, the comparisons revealed that the polymerase domains of the two RT structures are almost identical. Orientations of two key positively charged residues (Arg-72 and Lys-65) that interact with the β- and γ-phosphates of the incoming dNTP are also very similar. On the other hand, protonation of the PPi leaving group is a prerequisite for its release (10). The proton donor to the PPi leaving group in HIV-1 RT was suggested to be Lys-220 (54), but that may facilitate chemistry, not PPi release. Orientations of this key positively charged residue might be different with a DNA duplex or a DNA/RNA hybrid. However, a direct comparison is infeasible as Lys-220 is positioned too far away to readily interact with the triphosphate moiety of the incoming nucleotide, as its “real” position is probably influenced by artifacts from cross-linking of the enzyme to DNA (54) necessary to obtain crystal structures. Consequently, the mechanistic basis for rate-limiting PPi release observed with an RNA template remains a mystery. Although the structure of HIV-1 RT co-crystallized with the phosphonofumaric acid (or foscarnet), a PPi mimic) has recently been available (55), little information regarding PPi binding and dissociation-associated conformational isomerization can be extracted from the snapshot. Time resolved X-ray crystallography monitoring the course of phosphodiester bond formation and concomitant PPi release by HIV-1 RT in complex with a DNA/RNA hybrid may unravel this mystery.

Because of the relatively slow PPi release, the forward polymerization kinetic data revealed the reversibility of the chemical reaction at the active site, yielding a reverse rate constant for chemistry \( k_{-2} \) as 3.5 s\(^{-1}\). Considering the forward rate of chemistry \( k_j \) of 250 s\(^{-1}\), the chemical reaction is highly favorable, which can be attributed to two mechanisms. First, the catalytic Mg\(^{2+}\) ions A and B dissociate from the active site after the formation of the phosphodiester bond and are unable to rebind in the absence of PPi, or next cognate nucleotide (55). Secondly, PPi molecules are generally protonated in solution, but deprotonation of PPi, is not easy as Lys-220 of HIVRT (the general acid for catalysis) can be quickly reprotonated by solvent after PPi release (54).

Notably, a new kinetic partitioning between product release and the reverse chemistry was identified. Similarly, this partitioning strongly favors the forward product release \( k_j \gg k_{-2} \) and, therefore, does not contribute to nucleotide selectivity. In the accompanying paper, our data also showed that the relatively slow PPi release plays an important role to coordinate the polymerase and RNAase H active sites of HIVRT during RNA-dependent DNA synthesis (24).

The most remarkable observation reported here is the exceedingly slow dissociation of PPi, after incorporation of an incorrect nucleotide (dGTP:pA). To get the best estimates for the kinetic parameters governing the incorporation of dGTP, the data obtained over different timescales were fit simultaneously (Fig. 7) according to the model shown in Scheme 4. The simple model adequately accounts for the observed dependence of the amplitude of the burst phase on dGTP concentration. Similar concentration dependence has been observed during 8-oxodG incorporation by human mitochondrial DNA polymerase, implying that binding of 8-oxodG is reversibly linked to product formation (20). However, the chemical reaction is not able to come to equilibrium if PPi release is fast and largely irreversible, suggesting that PPi release must be slow after incorporation of an 8-oxodG. Subsequent analysis of AZT incorporation revealed the same phenomena, and direct measurements of PPi dissociation following AZT incorporation yielded an extremely slow off-rate (3, 21).

An alternative explanation for our observations could be that PPi release was readily reversible. To test this possibility we examined the kinetics of the reverse reaction using a mismatched dGMP-terminated primer strand. To our surprise no pyrophosphorolysis activity was observed. The result revealed that HIVRT was able to catalyze the reverse reaction only when a naturally terminated primer strand was used (Fig. 2E). Little is known about conformational changes of HIVRT during pyrophosphorolysis due to the lack of corresponding structural information. However, the fact that we were not able to force the reverse reaction to take place by adding relatively high concentration of PPi to the RT-DNA/RNA complex (primer terminated with dGMP) suggests that when dGMP is in a mismatch with the template strand, PPi is unable to rebind or productively associate with the RT-DNA/RNA complex with sufficient binding energy to organize the active site to promote pyrophosphorolysis. These results argued directly against the hypothesis that a substantial reverse rate constant for pyrophosphate binding and reaction was the cause of the abnormal kinetics of dGTP incorporation. Rather, the data suggest that slow PPi release is limited by the enzyme opening after incorporation of a mismatch, which provides an additional check for improving enzyme fidelity. The slow PPi release provides additional time for the reversal of chemistry and release of the bound mismatched nucleotide.

The effect of slow PPi release on the efficiency of dGTP incorporation by HIVRT is quite large. The commitment to forward catalysis is not made until PPi dissociates from the enzyme, indicating that the specificity constant for incorporation will be correspondingly low. A calculation of \( k_{cat}/K_m \) from intrinsic rate constants (Table 4) found this to be the case and dictates that previous calculation of the discrimination \( D = (k_{cat}/K_m)_TTP/ (k_{cat}/K_m)_dGTP \) against dGTP must be revised to \((2.4 \pm 0.7) \times 10^6\), a value >20-fold greater than the number would be if the slow PPi step was ignored. Similarly, in the case of AZT and 8-oxodG incorporation by human mitochondrial DNA polymerase, slow PPi release allows for the direct reversal of the chemistry step and reduces \( k_{cat} \) for incorporation (20, 21). This represents a novel mechanism by which a polymerase can increase discrimination against an undesirable substrate.

The 20-fold increase in discrimination that we observed for HIVRT may also fill the gap between the fidelity of HIV-1 RT measured by in vitro (16, 29, 35–40) and in vivo approaches (41–43). Further test of other combinations of misincorporation might not be necessary as presteady-state kinetics studies of fidelity by HIVRT (16) and human mitochondrial DNA polymerase (56) revealed comparable levels of discrimination.
against other incorrect base pair combinations with the only exception of dGTP:dT (or TTP:dG) that showed much lower discrimination. Further studies will need to be performed to evaluate the extent to which the slow PPi release would increase the fidelity of HIV-1 RT during plus strand DNA synthesis as unequal error rates were observed with RNA and DNA templates (37).

Currently, we have no direct measurement to demonstrate that the slow phase in Fig. 7B corresponds to the slow PPi release step, although it is consistent with the observed nucleotide dependence of the amplitude of the reaction. The coupled stopped-flow assay was performed to measure the rate of PPi dissociation using dGTP as the substrate. However, a considerable overestimation of the rate was likely obtained from the data because the presence of the phosphate mock in the assay would scavenge traces of phosphate with a faster rate than its release from the reaction and, therefore, result in reduced amplitude (57). Nonetheless, the inability of our coupled stopped-flow assay to measure PPi release kinetics during dGTP incorporation suggested that the off-rate of PPi is even slower than Pi consumption by the mop, which is $\sim 0.01 \text{ s}^{-1}$ (46). This number is consistent with the rate obtained from global fitting of data shown in Fig. 7 (0.003 s$^{-1}$).

In summary, here we show for the first time that PPi release limits the rate of processive RNA-dependent DNA synthesis and improves the overall fidelity of the enzyme. These findings enriched our understandings of the kinetic basis for the distinctions between rate-determining versus specificity-determining steps (58) and allow estimates for the rate of reversal of the chemistry step at the active site of the enzyme. Accordingly, these data provide the first measurement of the equilibrium constants governing polymerization and the first complete free energy profile for HIVRT.

**Experimental Procedures**

**Expression and Purification of Unlabeled and MDCC-labeled HIV Reverse Transcriptase**—Unlabeled WT HIVRT protein was expressed and purified following methods previously described (1, 2). Briefly, the two subunits of WT RT were individually expressed in T7 Express Competent *E. coli* (New England BioLabs), mixed in a 1:1 ratio and purified by tandem Q-Sepharose and Bio-Rex 70 columns followed by a single-strand DNA affinity column. The MDCC-labeled protein was expressed and purified following the same procedure except that an additional chromatographic step with Bio-Rex 70 column was performed to remove excess MDCC after the labeling reaction. The enzymes were assayed by presteady-state burst experiments to determine the active site concentrations, divided into aliquots, rapidly frozen, and stored at $80^\circ$C.

**DNA and RNA Substrates for Kinetic Studies**—DNA primers and templates (Table 1) were ordered from Integrated DNA Technologies (IDT) and purified by gel extraction. The 45-nt RNA templates with semi-random sequence (r45) or containing a string of Gs (r45$_{\text{pG}}$) were ordered from IDT with RNase-free HPLC purification. Dideoxy-terminated DNA primer (d25$_{\text{ad}}$) was made through enzymatic synthesis using RT and purified by gel extraction. Primers used in quench flow assays were 5‘-$^{32}$P-labeled using T4 polynucleotide kinase (New England BioLabs). Annealing of primers and templates was carried out by mixing the two oligonucleotides at a 1:1.2 molar ratio (excess template) and incubating at 95 °C for 5 min (DNA primers and RNA templates were incubated at 67 °C for 8 min) followed by slow cooling to room temperature.

**Magnesium Concentrations**—All experiments were performed in a buffer containing 50 mM Tris, pH 7.5, 100 mM KCl, and 0.1 mM EDTA at 37 °C. In quench-flow and stopped-flow experiments, 0.1 mM EDTA was included in the preincubation when forming the enzyme-DNA duplex complex, and 10 mM MgCl$_2$ was added with nucleotides or PPi to start the reaction.

**Quench Flow Kinetic Assays**—Kinetics of nucleotide incorporation were measured by rapidly mixing a preformed enzyme-p/t complex (generally 175 nM WT_HIVRT and 75 nM primer/template) with various concentrations of nucleotide using a KinTek RQF-3. Substrates (nucleic acids and nucleotides) and RT proteins (labeled or unlabeled) vary as specified in the corresponding text and figure legends. Kinetics of pyrophosphorolysis were measured by hand mixing methods in which a pre-formed enzyme-DNA/RNA complex (100 nM MDCC-labeled WT_HIVRT and 150 nM d26/r45) was mixed with various concentrations of PPi. Processive nucleotide incorporation was measured by rapidly mixing a preincubated enzyme-p/t complex (175 nM WT_HIVRT and 75 nM primer/template) with 100 μM concentrations of each nucleotide as specified in the text and corresponding figure legends.

**Stopped Flow Kinetic Assays**—Stopped flow assays were performed using a KinTek AutoSF-120x to measure the kinetics of enzyme conformational changes and the rates of TTP binding and incorporation using MDCC-labeled HIVRT as described previously (1). Various concentrations of TTP were rapidly mixed with a preformed enzyme-DNA/RNA complex (100 nM MDCC-labeled WT_HIVRT and 150 nM d25/r45), and the reactions were monitored by stopped-flow fluorescence methods. Similar experiments were performed at lower temperatures (5, 10, 15, 20, and 25 °C), and the data were fit globally to estimate the rate of fingers closure at 37 °C, which was too fast to measure directly. To determine the nucleotide dissociation rate, 200 nM enzyme-DNA$_{\text{ad}}$/RNA (200 nM MDCC-labeled WT_HIVRT and 300 nM d25$_{\text{ad}}$/r45 (Table 1)) was preincubated with 2 μM TTP, then the preformed ternary complex was diluted 1:1 as it was mixed with 1 μM unlabeled enzyme-DNA/RNA duplex to serve as a trap for free nucleotide. Because we modeled the reaction explicitly, a large excess of trap was not required. The change in fluorescence of the labeled enzyme provided the rate of opening and nucleotide release. A coupled fluorescence assay described elsewhere (46) was used to measure the kinetics of pyrophosphate (PPi) release. Briefly, the preformed enzyme-DNA/RNA duplex (200 nM MDCC-labeled WT_HIVRT and 300 nM d25/r45) was rapidly mixed with 1 mM TTP in the presence of 0.6 μM pyrophosphatase, including the P$_i$ “mop” containing 0.1 mM 7-methylguanosine and 0.2 IU/ml purine nucleoside phosphorylase, as well as 0.5 μM concentrations of fluorescently labeled *E. coli* PB. Fluorescence was observed by excitation of MDCC at 425 nm and monitoring emission with a 475-nm band-pass filter with a 50-nm bandwidth. The signal from the MDCC-labeled PB was significantly larger than that from the MDCC-labeled HIVRT, so that
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the latter could be neglected. Control experiments were also performed using DNA template or unlabeled RT protein under specified conditions.

Global Data Fitting—Data within each figure were fit globally based on numerical integration of rate equations using KinTek Explorer software (59). For fitting fluorescence transients, extinction coefficients for each species were included as variables in the data fitting as described previously (1). Confidence contour analysis (23) was used to investigate whether the parameters were well constrained by the data. In the current studies the upper and lower limits derived from the confidence contour analysis were approximately symmetrical so we only reported ±S.E.

Author Contributions—A. L. designed and performed all of the experiments except the nucleotide dissociation and pyrophosphorylation experiments, which were performed by S. G. A. L. wrote the first draft of the paper. K. A. J. assisted with the design and interpretation of the experiments and in the writing of the paper.

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