Pre-Steady-State Kinetic Analysis of a Family D DNA Polymerase from Thermococcus sp. 9°N Reveals Mechanisms for Archaeal Genomic Replication and Maintenance *

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*Running Title: Kinetics of a Family D DNA polymerase

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Background: Family D DNA polymerase (polD) is important for replication in most archaea, excluding Crenarchaeota.

Results: We report a detailed kinetic characterization of polD nucleotide incorporation, mismatch discrimination and 3'→5' exonuclease hydrolysis.

Conclusion: Despite evolutionary divergence, polD kinetic pathways share similarities to other DNA polymerase families.

Significance: This work contributes to unifying our understanding of DNA polymerase function.

ABSTRACT

Family D (polD) DNA polymerases have been implicated as the major replicative polymerase in archaea, excluding the Crenarchaeota branch, and bear little sequence homology to other DNA polymerase families. Here we report a detailed kinetic analysis of nucleotide incorporation and exonuclease activity for a Family D DNA polymerase from Thermococcus sp. 9°N. Pre-steady-state single-turnover nucleotide incorporation assays were performed to obtain the kinetic parameters, $k_{pol}$ and $K_d$, for correct, incorrect, and ribo-nucleotide incorporation by exonuclease deficient polD. Correct nucleotide incorporation kinetics revealed relatively slow maximal rate of polymerization ($k_{pol} \sim$2.5 s⁻¹) and especially tight nucleotide binding ($K_{d}^{dTTP} \sim$1.7 μM), compared to DNA polymerases from Families A, B, C, X, and Y. Furthermore, pre-steady-state nucleotide incorporation assays revealed polD prevents the incorporation of incorrect and ribo-nucleotides primarily through reduced nucleotide binding affinity. Pre-steady-state single-turnover assays on wild-type 9°N polD were used to examine 3'→5' exonuclease hydrolysis activity in the presence of Mg²⁺ and Mn²⁺. Interestingly, substituting Mn²⁺ for Mg²⁺ accelerated hydrolysis rates over 40-fold ($k_{exo} \geq$110 s⁻¹ versus ≥2.5 s⁻¹). Preference for Mn²⁺ over Mg²⁺ in exonuclease hydrolysis activity is a property unique to the polD family. The kinetic assays performed in this work provide critical insight into the mechanisms polD employs to accurately and efficiently replicate the archaean genome. Furthermore, despite the unique properties of polD, this work suggests a conserved polymerase kinetic pathway is present in all known DNA polymerase families.

INTRODUCTION

DNA polymerases play central roles in genome replication, maintenance and repair, and are therefore critical for genome integrity. Consequently, DNA polymerases have been the subject of extensive and widespread research for over 60 years (1). Multiple sequence alignment studies have classified DNA polymerases into 7 different families: A, B, C, D, X, Y, and reverse transcriptases (RTs) (2–4). Kinetic studies of Family A, B, C, X, Y, and RT DNA polymerases have proven to be a powerful tool in understanding polymerase function (5–7). Importantly, such studies reveal the kinetic basis of nucleotide selection and mismatch discrimination mechanisms, offering critical insight into how DNA polymerases accurately and efficiently synthesize and maintain genomes.
While the majority of DNA polymerase families have been well characterized, studies of Family D DNA polymerases (polD), found in all known archaea, (excluding the crenarchaeota branch), have been limited (8). Previous polD characterization studies have revealed that this polymerase is heterodimeric, comprised of a large polymerase subunit and small 3'-5' exonuclease proofreading subunit (9-11). The activity of the two subunits is co-dependent and the presence of both is required for activity of either unit, a feature unique to the polD family (12,13). Gene deletion studies in Thermococcus kodakarenisis (Tko) and Methanococcus maripaludis (Mma) suggest that polD is essential for cell viability and indicate that polD may be the major replicative polymerase responsible for leading and lagging strand synthesis in these organisms (14,15). Furthermore, polD forms complexes with many replisome components, including the minichromosome maintenance (MCM) helicase, proliferating cell nuclear antigen (PCNA) processivity factor and DNA ligase, providing further support that polD is a replicative polymerase in archaea (15-18). It is still unclear what specific role(s) polD plays in archaeal genome replication and which roles are played by other DNA polymerases. For example, efficient Okazaki fragment maturation is dependent on a Family B DNA polymerase (19).

The biochemical properties of a well-expressed, highly soluble polD from Thermococcus sp. 9°N have been characterized in our lab (20). In this work, a qualitative assessment of 9°N polD 3'-5' exonuclease activity showed a dependence on Mg²⁺ or Mn²⁺ for catalytic activity, with a preference for Mn²⁺ (20). Despite its 3'-5' exonuclease, recombinant polD has relatively low fidelity compared to most other characterized DNA polymerases (20).

To gain further insight into this unique multi-subunit polymerase, we have performed a detailed kinetic characterization of the polymerase activity of an exonuclease deficient mutant of polD, including kinetics of correct nucleotide incorporation and pyrophosphorylation as well as incorrect nucleotide and ribonucleotide discrimination kinetics. Furthermore, we kinetically characterized the 3'-5' exonuclease activity of wild type (WT) polD at 60 °C in the presence of Mn²⁺ and Mg²⁺. This detailed kinetic analysis performed on a Family D DNA polymerase, provides insight into polymerase and exonuclease reaction pathways, and suggests mechanisms for archaeal genome maintenance.

EXPERIMENTAL PROCEDURES
Enzymes, oligonucleotides and reagents—WT Thermococcus sp. 9°N polD and an exonuclease deficient polD mutant (H554A) (abbreviated as polD for clarity) were expressed and purified as previously described (20). PoID small and large subunits form a heterodimeric complex in an approximately 1:1 molar ratio as determined by SDS-PAGE gel analysis and quantitation by densitometry (data not shown). Oligonucleotides used in this study were purchased from Integrated DNA Technologies (IDT, Coralville, IA) (Table 1). For oligonucleotide detection, the primer strand was 5' labeled with a 5-carboxyfluorescein (FAM) fluorophore (IDT). Buffers used in this study were from New England Biolabs (NEB, Ipswich, MA).

PoIDsteady-state single nucleotide incorporation assay— A steady-state kinetic assay was performed to determine if polD followed burst kinetics and to calculate an active enzyme concentration. The primer-template used to monitor steady-state kinetics was prepared by annealing the 50mer 5'-FAM primer (10 µM) (Table 1) to the 62mer “C Template-1” (15 µM) (Table 1) in 1X ThermoPol buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100, pH 8.8 at 25 °C) by heating to 95 °C for three minutes following by cooling to room temperature. A 250 µl polD−/DNA aliquot was prepared by mixing Thermopol buffer (1X final concentration), primer/template DNA (80 nM final concentration) and polD− (20 nM final concentration) to ensure a 4-fold excess of DNA to polD. A second 250 µl aliquot was prepared by mixing Thermopol buffer (1X final concentration) and dGTP (200 µM final concentration). Using a Rapid Chemical Quench apparatus (RQF) (KinTek Corp., Snow Shoe, PA) set to 62.5 °C with a circulating water bath to achieve a final 60 °C reaction temperature, the polD−/DNA and dGTP solutions were rapidly mixed from 0.07 – 10 s and quenched with 50 mM EDTA. After mixing of equal volumes of polD−
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/ DNA and dGTP solutions by the RQF, the final reaction concentrations were 40 nM DNA, 10 nM polD and 100 µM dGTP in 1X ThermoPol buffer. A negative control reaction was performed in which the dGTP aliquot was replaced with 1X ThermoPol buffer and reacted in the RQF with polD/DNA for 10 seconds. To ensure that the DNA substrate (40 nM) was in molar excess to satisfy steady-state requirements, control experiments were performed with a fixed polD− concentration (10 nM final concentration) and varying DNA concentrations (10 – 80 nM final concentration) as described above (data not shown). Furthermore, the dependence of burst amplitude on enzyme amount was confirmed (data not shown). Reaction products were separated by capillary electrophoresis using a 3730xl Genetic Analyzer (Applied Biosystems) and fluorescent peaks were analyzed using Peak Scanner software version 1.0 (Applied Biosystems). The concentration of product (51 nt DNA with a FAM label) was graphed as a function of time and the data were fit to burst equation 1 using the nonlinear regression program Kaleidagraph (Synergy Software). From the fit, one can extract the active enzyme concentration (A), the initial rate of product formation (kobs) and steady-state turnover rate (k2), which is obtained by dividing k2 by A.

\[
[\text{Product}] = A[1-\exp(-k_{\text{obs}}t)] + k_2t \quad (1)
\]

All kinetic assays described within this text were performed at least twice to ensure experiment reproducibility.

PolD pre-steady-state single nucleotide incorporation— To obtain the rates of correct, incorrect, and ribo- nucleotide incorporation by polD, pre-steady-state single nucleotide assays were performed. The primer-template substrates used in these assays were prepared as described above. A 150 µl polD/DNA aliquot was prepared by mixing ThermoPol buffer (1X final concentration), primer/template DNA (30 nM final concentration), and a 3-fold excess of polD− (90 nM active enzyme final concentration); a control experiment demonstrated polymerase saturation was reached at 3-fold excess polD−, satisfying pre-steady-state requirements (data not shown). A second 150 µl aliquot was prepared by mixing ThermoPol buffer (1X final concentration) and dNTP (5 - 200 µM). Higher concentrations (100 - 4000 µM) were required for incorrect nucleotide and ribonucleotide incorporation assays. Using the RQF instrument, polD/DNA construct was rapidly mixed with dNTP and quenched with 50 mM EDTA. After mixing an equal volume of polD/DNA and dNTP solutions by the RQF, the final reaction concentrations were 15 nM DNA, 45 nM active polD and 2.5 - 100 µM dNTP for correct nucleotides (or 50 - 2000 µM dNTP for incorrect or ribo- nucleotides) in 1X ThermoPol buffer. A control reaction was performed in which dNTP was replaced with 1X ThermoPol buffer and reacted in the RQF with polD/DNA for 10 seconds. Reaction products were separated by capillary electrophoresis as described above.

Because reaction products for dCTP incorporation did not resolve into two fully distinguishable substrate and product peaks by capillary electrophoresis, we instead separated the products of dCTP insertion by denaturing gel electrophoresis (20% polyacrylamide, 8 M urea, 1X TBE buffer), visualized by Typhoon TRIO (GE Healthcare) and quantitated with ImageQuant software (Molecular Dynamics). In order to confirm that both capillary and gel electrophoresis gives comparable results, the 250 µM dTTP incorporation time course was analyzed by both capillary electrophoresis and gel electrophoresis (data not shown).

The product concentration was graphed as a function of time and the data were fit to the single-exponential equation 2 to obtain the observed rate constant of nucleotide incorporation (kobs) using the nonlinear regression program Kaleidagraph (Synergy Software).

\[
[\text{Product}] = A[1-\exp(-k_{\text{obs}}t)] \quad (2)
\]

To obtain the maximum rate of polymerization constant (kpol) and apparent equilibrium dissociation constant (Kd dNTP), the kobs were graphed as a function of dNTP (or rNTP) concentration and the data were fit to the hyperbolic equation 3 using Kaleidagraph.

\[
k_{\text{obs}} = k_{\text{pol}}[\text{dNTP}]/(K_d + \text{dNTP}) \quad (3)
\]

The specific activities for correct, incorrect, and ribo- nucleotide incorporation were calculated...
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using equation 4, and the nucleotide selectivity for incorrect and ribo-nucleotide incorporation was calculated using equation 5.

\[
\text{Specific Activity} = \frac{k_{\text{pol}}}{K_d} \quad (4)
\]

Nucleotide Selectivity = \frac{(k_{\text{pol}}/K_d)_{\text{correct}}}{(k_{\text{pol}}/K_d)_{\text{incorrect}}} \quad (5)

PolD pyrophosphorolysis- Under certain circumstances, DNA polymerases can perform the reverse reaction (pyrophosphorolysis) to remove nucleotides from the 3'-end of the DNA molecule in the absence of exonuclease activity. To obtain the rate of polD− pyrophosphorolysis, a 3-fold excess of polD− was preincubated with 5'-FAM primer/C Template-1 and was rapidly mixed with 50 − 500 µM (final concentration after RQF mixing) inorganic phosphate (PPi) using the RQF at 60 °C. Reaction products were separated by capillary electrophoresis and analyzed as described above. The concentration of pyrophosphorolysis product (<50 nt DNA) was graphed as a function of time and fit to equation 2 to obtain the observed rate constants \((k_{\text{obs}})\), followed by fitting the \(k_{\text{obs}}\) as function of PPi concentration in equation 3 to obtain a maximal rate for pyrophosphorolysis \((k_{\text{pyro}})\) and apparent equilibrium dissociation constant of PPi \((K_{d,\text{PPi}})\). In reactions with multiple pyrophosphorolysis events, all product peaks were summed as total product.

PolD 3'-5' exonuclease activity- The kinetics of polD 3'-5' exonuclease activity was measured by monitoring shortening of a FAM-labeled DNA primer. To obtain the rate of 3'-5' exonuclease activity on single-stranded DNA, a pre-steady state single-turnover assay was performed. A 40 nM aliquot of 5'-FAM primer was prepared in 1X ThermoPol II buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 0.1% Triton X-100, pH 8.8 at 25 °C) supplemented with either 2 mM MgSO₄ or MnSO₄. A second aliquot containing polD (120 nM) was prepared in 1X ThermoPol II buffer supplemented with either 2 mM MgSO₄ or MnSO₄. Using the RQF instrument, polD and 5'-FAM labeled primer were rapidly mixed at 60 °C from 0.002 − 5 s and quenched with 0.1 N H₂SO₄. Final reaction conditions after mixing by the RQF were 20 nM primer, 60 nM polD in 1X ThermoPol II buffer with 2 mM MgSO₄ or MnSO₄. After quenching, samples were neutralized with 1 N NH₄OH and reaction products were separated by capillary electrophoresis and analyzed by peak scanner as described above. All product peaks (<50 nt) resulting from 3'-5' exonuclease hydrolysis were summed and graphed as a function of time followed by fitting data to equation 2 as previously described. It is important to note that 0.05, 0.1, and 1 M EDTA did not sufficiently quench 3'-5' exonuclease reactions at rapid time scales but 0.1 N H₂SO₄ was sufficient to quickly and reliably quench activity (data not shown).

RESULTS

Analysis of polD− multiple turnover steady-state nucleotide incorporation kinetics reveals a post-chemistry rate limiting step – Steady-state kinetic assays are used to infer the rate-determining step of a catalytic pathway and are performed under multiple turnover conditions, i.e. [substrate] >> [enzyme]. Previous steady-state kinetic studies performed on a host of different DNA polymerases reveal nucleotide incorporation is fast and DNA release is rate limiting (21-27). In order to determine if the same is true for polD−, a steady-state multiple turnover assay was performed in which polD− was preincubiated with a 4-fold excess of DNA and rapidly mixed with dGTP at 60 °C using an RQF instrument. Under these conditions, only one dGTP is added to the primer because the next position requires incorporation of a nucleotide that is absent in the reaction mixture. PolD− must then dissociate from the DNA and bind to a new primer/template to repeat the addition of the single dGTP. DNA substrate and product were analyzed by capillary electrophoresis. Concentration of the 51 nt DNA product was graphed as a function of time and the steady-state kinetic parameter, \(k_{\text{obs}}\), was determined. We observe a rapid initial accumulation of product, designated the burst phase, followed by a slower linear phase of product formation (Figure 1). The burst phase represents the fast chemistry step of nucleotide incorporation and is proportional to the concentration of the active pre-bound polymerase. The linear phase represents a slow-step occurring after chemistry. This slow phase, likely dominated by the rate of DNA release between nucleotide addition reactions,
defines the polD− steady-state turnover rate, 0.3 s⁻¹ (Table 2). Furthermore, by extrapolating the linear phase through the y-axis, the concentration of active enzyme can be determined. For this preparation of polD−, 4.8 nM of the 10 nM was active. This active enzyme concentration (4.8 nM) was used to calculate active polD− concentration in subsequent pre-steady-state studies using this preparation of enzyme.

Several factors may account for the discrepancy between total and active enzyme concentrations. The concentration of polD was first determined spectrophotometrically, which may account for inaccurate apparent protein concentration. Alternatively, incorrect stoichiometry between small and large subunits, misfolding of the subunits or multiple DNA polymerases binding to a single DNA substrate may also account for a low apparent active enzyme concentration.

**Analysis of polD− pre-steady-state single-turnover correct nucleotide incorporation kinetics reveals a slow rate of incorporation and tight nucleotide binding.** Pre-steady-state kinetic assays are performed under single-turnover conditions, i.e. [enzyme] >> [substrate], and are used to determine the kinetic parameters associated with steps masked by the rate limiting step, such as single nucleotide incorporation (7). To obtain rates for nucleotide incorporation, including maximal rate of polymerization, $k_{pol}$, and apparent equilibrium dissociation constant, $K_d$ for dNTP, polD− pre-steady-state kinetic assays were performed. Such parameters provide insight into how polymerases discriminate against incorrect nucleotides and incorporate correct nucleotides.

The $k_{pol}$ reflects how fast the polymerase will incorporate a nucleotide, while the $K_d$ reflects how tightly the polymerase binds a nucleotide, wherein a lower $K_d$ reflects tighter nucleotide binding. Although $k_{pol}$ and $K_d$ can be obtained from the burst phase of steady-state kinetics, such methods may result in parameters with large sources of error (5). Therefore to measure pre-steady-state kinetics, a 3-fold excess of active polD− was preincubated with DNA, rapidly mixed with single dNTP solutions at 60 °C and analyzed as described above. A schematic of the incorporation assay and expected capillary electrophoresis results are depicted in Figure 2.

For each dNTP concentration, the concentration of product was graphed as a function of time to obtain the observed rate of nucleotide incorporation, $k_{obs}$. The $k_{pol}$ as well as the $K_d$ for each dNTP was obtained by graphing $k_{obs}$ versus dNTP concentration.

As shown in Figure 3A for dTTP incorporation paired with template dA (dTTP:A), we observe an increase in $k_{obs}$ with increasing dTTP concentration, where we reach a maximal $k_{obs}$ at high (100 µM) dTTP concentration. When $k_{obs}$ is graphed as a function of dTTP concentration, the maximal rate of polymerization is obtained from the plateau, while the $K_d$ is the dTTP concentration at half $k_{pol}$ (Figure 3B).

Table 3 shows the obtained $k_{pol}$ and $K_d$ values for correct nucleotide incorporation using separate matched templates for each possible dNTP. All values for $k_{pol}$ are within 2-fold, ranging from 1.8 – 3.1 s⁻¹, suggesting polD incorporates all correctly base-paired nucleotides at a similar rate. Similarly, the obtained $K_d$ values are within 3-fold, ranging from 0.9 – 2.5 µM, indicating that polD binds each correct nucleotide with similar affinity (Table 3). Furthermore, we calculated the specificity constant for each correct nucleotide, obtained by dividing $k_{pol}$ by the corresponding $K_d$ (Table 3). The specificity constant, a reflection of both the incorporation rate and dNTP binding, is used to compare the efficiency of incorporation for each nucleotide by a polymerase, as well as compare the efficiency of incorporation to other polymerases. A larger specificity constant reflects more efficient binding and incorporation (5). For polD, specificity constants for correct nucleotide incorporation range from 1.0 – 2.3 µM⁻¹ s⁻¹ (Table 3), again demonstrating the enzyme lacks preference amongst Watson-Crick base paired dNTP substrates.

**Pre-steady-state kinetic analysis of polD− incorrect nucleotide incorporation reveals the presence of a nucleotide discrimination mechanism.** DNA polymerases have evolved specific mechanisms to discriminate against the incorporation of incorrect nucleotides during synthesis [reviewed in reference (4)]. Such mechanisms are critical for faithful replication of the genome and to ensure the transfer of accurate information to subsequent generations. Importantly, nucleotide selectivity mechanisms
have not been characterized in polD. Therefore, we performed a thorough kinetic characterization of incorrect nucleotide incorporation to understand polD fidelity. Pre-steady-state single-turnover kinetic experiments were performed on the 12 possible incorrect base pairing combinations (Table 3).

As shown in Figure 3C for dATP:A incorporation for dATP incorporation paired with template dA, $k_{obs}$ increases with higher dATP concentrations. It is important to note that maximal $k_{obs}$ for dATP:A incorporation is reached at exceedingly high dATP concentration, 2000 μM, compared to 100 μM for correct dTTP:A incorporation. Furthermore, maximal product incorporation is achieved after 60 seconds for incorrect dATP:A incorporation, 12 times slower than for correct dTTP:A incorporation. By plotting the observed $k_{obs}$ versus dATP concentration, the $k_{pol}$ and $K_d^{dNTP}$ for incorrect nucleotide incorporation were obtained (Figure 3D, Table 3). These results suggest that for misincorporation, high substrate concentrations and longer reaction times are required to drive the incorporation of the incorrect nucleotide, and imply polD contains specific mechanisms to exclude incorrect nucleotides, similar to other DNA polymerases.

Table 3 shows $k_{pol}$ and $K_d^{dNTP}$ values for all 12 incorrect nucleotide incorporation combinations. Obtained $k_{pol}$ rates range from 0.07 – 1.3 s$^{-1}$, resulting in a 1.5 – 45-fold decrease in $k_{pol}$ between correct to incorrect dNTP incorporation (Table 3). The $K_d^{dNTP}$ ranges from 300 – 1400 μM, a 120 – 1500-fold increase in $K_d^{dNTP}$ between correct and incorrect binding (Table 3). These results suggest that polD prevents the incorporation of incorrect nucleotides primarily through weaker binding of the incorrect nucleotide, as reflected in the large increase in $K_d$.

As done for correct nucleotide incorporation assays, the specificity constant was calculated for incorrect nucleotide incorporation. Nucleotide selectivity, obtained by dividing the specificity constant for the correct nucleotide by the specificity constant for the incorrect nucleotide, reflects overall DNA polymerase fidelity (5). Of the 12 potential mismatches, polD discriminates most strongly against a dGTP:G mismatch (8,100-fold) and weakly discriminates against a dATP:C mismatch (1,000-fold) (Table 3).

**Analysis of polD single-turnover ribonucleotide incorporation kinetics reveal the presence of a ribonucleotide discrimination mechanism**. In addition to incorrect nucleotide discrimination, polymerases discriminate against ribonucleotide incorporation (28,29). Excluding rNTPs is especially important due to the intracellular excess of rNTPs over dNTPs (30). Furthermore, rNTPs incorporated during replication may lead to genome instability by inducing strand breaks. Therefore, to understand if and how polD discriminates against ribonucleotides, a pre-steady-state single-turnover assay was performed examining rATP incorporation paired to dT (rATP:T) as described above. For rATP incorporation, $k_{pol}$ was 0.16 s$^{-1}$ and $K_d^{rNTP}$ was 360 μM, which corresponds to a 16-fold decrease in $k_{pol}$ and a 144-fold increase in $K_d^{rNTP}$ compared to incorporation of dATP (Table 4). Such results indicate ribonucleotide discrimination occurs primarily through reduced binding affinity of the rNTP. The specificity constant ($k_{pol}/K_d^{dNTP}$), 4.4 × 10$^3$, and nucleotide selectivity constant ($[k_{pol}/K_d^{dNTP}] / [k_{pol}/K_d^{rNTP}]$), 2.3 × 10$^3$, suggest dATP is incorporated 2,300-fold more efficiently than rATP (Table 4).

**Analysis of polD pyrophosphorolysis**- During the incorporation of dNTPs by a DNA polymerase, inorganic phosphate (PPI) is produced and released. If PPI stays bound within the enzyme active site, reversal of chemistry can occur resulting in pyrophosphorolysis, which shortens the primer and releases a dNTP. In the presence of a high concentration of PPI, the polymerase can remove multiple dNTPs during a single DNA polymerase:DNA binding event. Single-turnover kinetic assays were performed to test whether polD undergoes pyrophosphorolysis and to obtain the maximal rate of pyrophosphorolysis, $k_{pyro}$, and the apparent dissociation rate constant for PPI, $K_{d}^{PPI}$. A schematic of the pyrophosphorolysis assay, as well as the expected capillary electrophoresis results are shown in Figure 4. We observed pyrophosphorolysis of multiple nucleotides at longer time points (Figure 4B VI–VIII). Concentration of product (<50 nt DNA) was graphed as a function of time to obtain the observed rate constants, $k_{obs}$ (data not shown). To obtain $k_{pyro}$ and $K_d^{PPI}$, $k_{obs}$ was graphed as a
function of PPI concentration (Figure 4C). For polD, $k_{\text{pyro}}$ is 0.4 s$^{-1}$ and $K_d^{\text{PPi}}$ is 190 μM (Table 2).

**Analysis of polD 3'-5' exonuclease kinetics reveals a 40-fold preference for Mn$^{2+}$ over Mg$^{2+}$** – Along with nucleotide incorporation activity, many polymerases, including polD, contain a metal dependent 3'-5' exonuclease activity, which facilitates removal of incorrectly incorporated nucleotides, thereby increasing the overall fidelity of the polymerase (31). In vivo, a DNA polymerase melts duplex DNA to shuttle the single-stranded DNA primer to the exonuclease active site prior to hydrolysis (31). Importantly, melting of duplex DNA during DNA polymerase exonuclease hydrolysis was previously determined to be rate limiting (32). In order to focus on 3'-5' exonuclease hydrolysis rates rather than DNA duplex melting dynamics, experiments were designed using a single-stranded DNA substrate (32). To assess polD exonuclease activity in the presence of Mn$^{2+}$ and Mg$^{2+}$, we performed pre-steady state assays in which we rapidly mixed a 3-fold excess of polD with single-stranded 5'-FAM DNA in the presence of either metal ion. A schematic of this assay is shown in Figure 5A and representative CE traces for exonuclease kinetics done in the presence of Mn$^{2+}$ or Mg$^{2+}$ are shown in Figure 5B. Concentration of exonuclease product (< 50 nt DNA) was graphed as a function of time and fit to a single-exponential burst equation to obtain pre-steady-state kinetic rates of exonuclease hydrolysis (Figure 5). Due to rapid exonuclease hydrolysis by polD, multiple dNMPs were excised from the 5'-FAM substrate at each reaction time. We therefore report a lower limit for polD $k_{\text{exo}} \geq 110$ s$^{-1}$ and $\geq 2.5$ s$^{-1}$ in the presence of Mn$^{2+}$ and Mg$^{2+}$, respectively. The results obtained here suggest there is a $\geq 44$-fold increase in the exonuclease hydrolysis rate in the presence of Mn$^{2+}$ compared to Mg$^{2+}$ (Table 2). Several alternative RQF experiments were unsuccessful in limiting polD exonuclease hydrolysis to removal of a single dNMP, including prebinding polD to 5'-FAM ssDNA and reaction initiation by addition of Mn$^{2+}$ or Mg$^{2+}$ in the presence of a large excess of trap DNA to prevent rebinding to the FAM-labeled DNA (data not shown).

**DISCUSSION**

**Family D DNA polymerase kinetic scheme** – The overall polymerization kinetic pathway is highly conserved amongst different DNA polymerase families (Figure 6, top path). In this pathway, DNA polymerase first binds to DNA (E•DNA$_a$), followed by dNTP binding (E•DNA$_a$•dNTP), which induces a conformational change from an “open” polymerase conformation to a “closed” conformation. Once in a closed conformation, the $\alpha$-phosphate of the bound dNTP is within close proximity to the 3'-OH of primer DNA. Here, nucleotide incorporation chemistry occurs when the 3'-OH of primer DNA attacks the $\alpha$-phosphate of bound dNTP, incorporating dNMP and generating PPI (E•DNA$_{a+1}$•dNTP). Following nucleotide incorporation, the polymerase undergoes two conformation changes to release PPI and then DNA (E+DNA$_{a+1}$). All steps within the polymerase kinetic scheme are reversible. Furthermore, it is generally agreed upon that the conformational changes are faster than chemistry, and DNA release is the slow rate-limiting step of the kinetic pathway (5).

PolD, however, is structurally and genetically unrelated to any other DNA polymerase family. Notably, there are few structural or functional studies for this family of polymerases in the literature to date. Thus caution must be applied in making assumptions about the polD reaction mechanism through analogy to other studied polymerases. To address this issue, we have performed a variety of kinetic assays to elucidate the polD reaction pathway and allow comparisons with other polymerase families. Our findings demonstrate that PolD does, in fact, follow a similar overall polymerization kinetic pathway as other families of DNA polymerases.

Under multiple turnover conditions, we observed a burst kinetic profile, indicating that polD shares the feature of fast nucleotide incorporation chemistry followed by a rate-limiting post-phosphoryl transfer reaction. Pre-steady-state kinetic experiments revealed the $k_{\text{pol}}$ is 1.8 – 3.1 s$^{-1}$ and the $K_{d}^{\text{dNTP}}$ is 0.9 – 2.5 μM (Table 2) for all correct nucleotide base pairing combinations. Furthermore, pre-steady-state pyrophosphorlysis kinetics revealed nucleotide incorporation is indeed reversible, providing further evidence that Family D DNA polymerases follow the generally observed cross-family
polD, for polD is one of the fastest for kinetically characterized replicative polymerases (Family X and Y) (Table 5). On the contrary, polD has one of the smallest, and therefore tightest, $k_d^{dNTP}$ of kinetically characterized polymerases (Table 5). Importantly, the resulting specific activity for polD, $~1.8 \, \mu\text{M}^{-1} \, \text{s}^{-1}$, is average compared to other replicative, repair and translesion polymerases (Table 5).

**Metal ion dependence of polD 3′-5′ exonuclease ssDNA hydrolysis**—The proposed 3′-5′ exonuclease ssDNA hydrolysis kinetic pathway for polD is presented in Figure 6, bottom path. Importantly, polD exonuclease hydrolysis requires a divalent metal ion for catalysis, typically Mg$^{2+}$, and the reaction is non-reversible at the 3′-5′ exonuclease active site.

Previously reported qualitative data on 9°N polD showed that 3′-5′ exonuclease activity was dependent on Mg$^{2+}$ or Mn$^{2+}$, with an increase in activity seen for Mn$^{2+}$ (20). The quantitative pre-steady-state exonuclease kinetics performed here reveal a $\geq 44$-fold increase in $k_{exo}$ in the presence of Mn$^{2+}$ versus Mg$^{2+}$ (Table 2). Similar to 9°N polD, the 3′-5′ exonuclease activity of polD from *Pyrococcus horikoshii* is more robust in the presence Mn$^{2+}$ over Mg$^{2+}$, while polD from *Methanococcus jannaschii* requires Mn$^{2+}$ and is inactive with Mg$^{2+}$ (13,33,34). Differences in metal requirements between species suggest that the 3′-5′ exonuclease active site of the polD small subunit may differ between different archaeal species.

The polD 3′-5′ exonuclease small subunit bears homology to the calcineurin-like phosphoesterase family (35-37). This calcineurin-like phosphoesterase superfamily requires the presence of two divalent metal ions per active site for catalysis, typically Mn$^{2+}$, Ni$^{2+}$, Ca$^{2+}$, Fe$^{2+}$, Fe$^{3+}$ or Zn$^{2+}$, and contains a wide array of members, including phosphoserine/threonine phosphodiesterases, nucleotidases and nucleases (38). The polD homology with calcineurin-like phosphoesterases and its preference for Mn$^{2+}$ over Mg$^{2+}$ suggest that the polD small subunit may at one time been an independently active exonuclease enzyme. Other replisome proteins including the GINS-associated nuclease (GAN), a ssDNA 5′-3′ exonuclease, rely upon Mn$^{2+}$ for exonuclease activity, with limited activity observed in the presence of Mg$^{2+}$ (17). Together these data suggest a putative role of Mn$^{2+}$ as a cofactor during archael replication. However, it is still unclear which metal ion, Mn$^{2+}$ or Mg$^{2+}$, is bound within the polD 3′-5′ exonuclease site *in vivo*. The *in vivo* concentrations of Mn$^{2+}$ and Mg$^{2+}$ as well as the relative binding affinities of the two metals in the 3′-5′ exonuclease active site (both unknown parameters) determine which metal is bound *in vivo*.

**Comparison of nucleotide selectivity amongst different polymerase families**—Polymerases have evolved highly specific mechanisms to ensure selection and incorporation of the correct nucleotide. The pre-steady-state nucleotide incorporation kinetics performed in this work confirm the presence of nucleotide discrimination mechanisms within polD and suggest discrimination is achieved through weak binding and slow catalysis of incorrect nucleotides. Due to the lack of polD structural characterization studies, it is not yet possible to easily identify active site determinants for nucleotide discrimination.

A quantitative assessment of incorrect nucleotide incorporation is reflected by nucleotide selectivity constants. Polymerases with high nucleotide selectivity are less likely to incorporate incorrect nucleotides, and are therefore less likely to introduce mutations. Therefore, high nucleotide selectivity (incorporation of correct vs. incorrect dNTP) is an important polymerase feature to ensure accurate genome replication. Table 6 compares the average nucleotide selectivity of several DNA polymerase families. Selectivity values range from as high as $2.7 \times 10^5$ in the replicative Family B RB69 DNA polymerase (39), to as low as $3.0 \times 10^3$ in the translesion Family Y
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Dpo4 DNA polymerase (40). PolD nucleotide selectivity is relatively low compared to other families, especially for a polymerase implicated in playing a replicative role, with an average nucleotide selectivity of $4.3 \times 10^5$. Likewise, the data obtained here correspond well to previously published fidelity data, which suggests polD has higher error rate than typical replicative polymerases (41). We suspect the low fidelity observed for $9^{\circ}$N polD is due to the slow rate of polymerization observed for correct nucleotide incorporation, $k_{pol}$ between $1.8 - 3.1 \ \text{s}^{-1}$, in conjunction with the small decrease observed in $k_{pol}$ from correct to incorrect nucleotide incorporation ($1.5 - 45 \ \text{fold}$). Although the large increase in $K_d$ from correct to incorrect nucleotide binding in $9^{\circ}$N polD (120 – 1500-fold) somewhat offsets the small change in $k_{pol}$, replicative polymerases typically have a significant fold change in both $k_{pol}$ and $K_d$ during nucleotide discrimination. It is possible, and quite likely, that the $k_{pol}$, and consequently nucleotide selectivity and fidelity, of polD is highly dependent upon the presence of replisome components, such as PCNA, which are absent in our in vitro studies.

Ribonucleotide discrimination- In the cell, rNTPs are present in a 1000-fold excess over dNTPs (30). To cope with this imbalance and to maintain genome integrity during replication and repair, DNA polymerases have evolved specific mechanisms to exclude rNTPs (29). Family A, B, and Y DNA polymerases and RTs exclude rNTPs by a clash between a conserved bulky side chain "steric gate" amino acid and the rNTP C2'-OH. In Family X DNA polymerases (pol\(\alpha\) and pol\(\beta\)) an active site backbone carbonyl clashes with the ribose C2'-OH, preventing rNTP incorporation (42,43). These clashes prevent the binding and incorporation of the rNTP (25,44-46). The kinetic basis of rNTP discrimination in Family A, B, and RT is due to weak binding (high $K_d$) and slower incorporation (low $k_{pol}$) compared to dNTPs. Family Y DNA polymerases bind rNTPs and dNTPs with similar affinity, and therefore discrimination occurs during catalysis ($>6000$-fold reduction in $k_{pol}$ for rNTPs) (47,48).

The kinetic analysis of rATP incorporation by polD performed here shows discrimination is primarily due to weak rATP binding (144-fold higher $K_d$) and slower incorporation (16-fold slower $k_{pol}$) compared to dATP. Kinetic data are similar to Family A, B, Y, and RT discrimination kinetics and suggests that a polD active site amino acid may block rNTP. However, the amino acid(s) important for rNTP discrimination in polD are currently unknown. PolD lacks conserved steric gate motifs (28,29) and in the absence of polD structural information, the location and identity of steric gate amino acids for rNTP discrimination within the polD family remain elusive.

Conclusions and future directions – The kinetics performed here on polD (the most recent DNA polymerase family to be identified) allows a comprehensive and direct comparison of nucleotide incorporation and 3'-5' exonuclease activities of all currently known DNA polymerase families. PolD employs nucleotide discrimination mechanisms to prevent misincorporations and contains an active site steric gate amino acid to prevent ribonucleotide incorporation, similar to other DNA polymerase families. Due to a lack of polD structural information, the identity of nucleotide discrimination and steric gate active site determinants remains unclear and will be the focus of future structural studies. Furthermore, structural data will help identify the polD active site, needed to explore the proposed conformational changes associated with nucleotide incorporation, including open and closed polymerase conformations. While polD has been implicated as the major replicative polymerase in much of archaea, the kinetic work performed here suggests low polymerase fidelity. We propose the presence of other replisome components, such as PCNA, will increase polD fidelity. The effect of these replisome components on polD fidelity will be explored in future work. Finally, while polD contains a 3'-5' exonuclease hydrolysis activity similar to many other polymerase families, the reliance on Mn\(^{2+}\) for optimal activity is unique to the polD family and importantly suggests that Mn\(^{2+}\) may play an important role in polD fidelity. Our data demonstrate that polD follows the same overall kinetic pathway as the other DNA polymerase families despite being a two-subunit enzyme with little sequence similarity to other families of DNA polymerases. Importantly,
despite active site divergence amongst families, evolved specific mechanisms to accurately and DNA polymerases, including polD, have each faithfully replicate genomes.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest with the contents of this article.

**AUTHOR CONTRIBUTIONS**

KMS and AFG designed research. KMS performed research. KMS and AFG analyzed data, wrote and approved the final version of the manuscript.

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FIGURE 1. PolD− steady-state dGTP incorporation. A 4-fold excess of 50 nt 5′-FAM primer/“C Template-1” was pre-incubated with polD− and rapidly mixed with dGTP followed by quenching with 50 mM EDTA using an RQF instrument at 60 °C. The yield of 51 nt product was graphed as a function of time and fit to a burst equation ([Product] = A[1-exp(-k OBS t) + k SAT]) to obtain a k OBS = k SAT / A of 0.3 s⁻¹, a k OBS of 2.8 s⁻¹ and an active enzyme concentration (A) (4.8 nM).

FIGURE 2. PolD− pre-steady-state dTTP incorporation. (A) Reaction scheme: A 50 nt 5′-FAM primer was annealed to template DNA and then pre-incubated with a 3-fold excess of active polD− followed by rapid mixing with dTTPs at 60 °C and incubation from 0 - 10 seconds. Since only dTTP is present, the primer can only be extended 1 nt unless an incorrect nt is added. (B) Substrates (50 nt) and incorporation products (51 nt) were resolved by capillary electrophoresis after denaturation of the double-stranded DNA.

FIGURE 3. PolD− pre-steady-state kinetics of correct and incorrect nucleotide incorporation. (A) A 50 nt 5′-FAM primer was annealed to “A Template-1” DNA and pre-incubated with a 3-fold excess of active polD− followed by rapid mixing (at 60 °C with 2.5 (●), 5 (■), 10 (◆), 25 (×), 50 (+) or 100 (▲) µM (final concentration) dTTP, creating correct T:A base pairing. Since only dTTP is present, the primer can only be extended 1 nt unless an incorrect nt is added. The 51 nt product was graphed as a function of time and fit to a burst equation ([Product] = A[1-exp(-k OBS t)]) to obtain k OBS. (B,D) The dependence of reaction rate k OBS on nucleotide concentration was fit to a hyperbolic equation ([k OBS] = k P O L[dNTP]/K D [dNTP]) to obtain k POL and K d[dNTP] for (B) correct dTTP:A and (D) incorrect dATP:A incorporation. (C) Primer/template DNA was prepared and pre-incubated with polD− as described above and mixed with 100 (■), 250 (◆), 500 (×), 1000 (+) or 2000 (▲) µM (final concentration) dATP, creating incorrect A:A base pairing, and fit to the above burst equation to obtain k OBS.

FIGURE 4. PolD− pyrophosphorolysis. (A) Reaction scheme: A 50 nt 5′-FAM primer was annealed to template DNA and pre-incubated with a 3-fold excess of active polD− followed by rapid mixing with PPi at 60 °C using an RQF instrument. Pyrophosphorolysis results in production of a smaller FAM labeled oligonucleotide. (B) Pyrophosphorolysis products (<50 nt) were resolved by capillary electrophoresis after DNA denaturation. (C) The dependence of reaction rate k OBS on PPi concentration was fit to a hyperbolic equation ([k OBS] = k P O L[dNTP]/K D + [dNTP]) to obtain the maximum rate of k PYRO and the equilibrium binding constant of K d−1, −0.4 s⁻¹ and −190 µM, respectively.

FIGURE 5. PolD 3′-5′ exonuclease kinetics. (A) Reaction Scheme: A 50 nt single-stranded 5′-FAM primer was rapidly mixed with a 3-fold excess of polD− in the presence of either MnSO₄ or MgSO₄ at 60 °C, and quenched with 0.1 N H₂SO₄ using an RQF instrument. (B) Expected capillary electrophoresis results for processive polD exonuclease hydrolysis in the presence of MnSO₄ or MgSO₄. (C) Product, <50 nt DNA, was graphed as a function of time and fit to a single-exponential burst equation ([Product] = A[1-exp(-k EXO t)]) to obtain the rate of polD exonuclease hydrolysis, k EXO, in the presence of MnSO₄ or MgSO₄, ~110 s⁻¹ and 2.5 s⁻¹, respectively.

FIGURE 6. Schematic of polD kinetic pathway. During polymerization (top pathway), DNA polymerase binds to DNA (E•DNAₐ), followed by dNTP binding (E•DNAₐ•dNTP). A proposed conformational change from an “open” polymerase conformation to a “closed” conformation, brings the α-phosphate of the bound dNTP within close proximity to the 3′-OH of the primer DNA. Here, nucleotide incorporation chemistry occurs when the 3′-OH of primer DNA attacks the α-phosphate of the bound dNTP, incorporating dNMP, extending the primer DNA by one nucleotide, and generating PPi (E•DNAₐ₊₁•PPi). Following nucleotide incorporation, the polymerase undergoes two proposed conformation changes to release PPi then DNA (E+DNAₐ₊₁). Alternatively, during 3′-5′ exonuclease hydrolysis (bottom pathway), the 3′-5′ exonuclease active site binds ssDNA (ssDNAₐ) and hydrolysis occurs, shortening the DNA to
release a dNMP from the enzyme/DNA complex (E•ssDNA_{n,1}). Kinetic constants derived from this study are averages from Table 3.
Table 1. Oligonucleotides Used to Study Thermococcus sp. 9°N polD Kinetics

| Name          | Sequence                      |
|---------------|-------------------------------|
| 5'-FAM primer | 5'-FAM-AGT GAA TTC GAG CTC GGT ACC CGG GGA TCC TCT AGA GTC GAC CTG CAG GT-3' |
| G Template-1  | 5'-CCC TAA TCA TAT CCT AGA CCT GCA GGT CCA ATG ATT CAC T-3' |
| A Template-1  | 5'-TTG CTC GTT TGC TGG GAA CCT GCA GGT CCA ATG ATT CAC T-3' |
| T Template-1  | 5'-AAG CAC GAA AGC AGG GCA GTG CCT GCA GGT CCA ATG ATT CAC T-3' |
| C Template-1  | 5'-AAG TAT GAA AGT AGG GCA CCT GCA GGT CCA ATG ATT CAC T-3' |
| G Template-2  | 5'-CCC TAA TCA TAT CCT TGA CCT GCA GGT CCA ATG ATT CAC T-3' |
| A Template-2  | 5'-TTG CTC GTT TGC TGG CAA CCT GCA GGT CCA ATG ATT CAC T-3' |
| T Template-2  | 5'-AAG CAC GAA AGC AGG GCA GTT CCT GCA GGT CCA ATG ATT CAC T-3' |
| C Template-2  | 5'-AAG TAT GAA AGT AGG GCA CCT GCA GGT CCA ATG ATT CAC T-3' |

*Bold letters indicate base opposite incoming nucleotide

*Underlined letters indicate change from Template 1 to Template 2

Table 2. Steady-State and Pre-Steady-State Kinetic Parameters of Thermococcus sp. 9°N polD

| Reaction Observed | Parameter | Value |
|-------------------|-----------|-------|
| DNA Release       | $k_{ss}$  | 0.30 ± 0.01 s$^{-1}$ |
| Polymerization    | $k_{pol}$ | 1.8 to 3.1 s$^{-1}$ |
|                   | $K_{d}$   | 0.9 to 2.5 μM |
| Pyrophosphorolysis| $k_{pyro}$| 0.40 ± 0.03 s$^{-1}$ |
|                   | $K_{d}$   | 190 ± 20 μM |
| ssDNA Exonuclease | $k_{exo}$ | ≥2.5 ± 0.3 s$^{-1}$ |
| 3'-5' Exonuclease | $k_{exo}$ | ≥110 ± 10 s$^{-1}$ |

*Polymerization $k_{pol}$ and $K_{d}$ values represent the ranges for all four dNTPs. Error is derived from the fit to the corresponding equation.
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Table 3. Pre-Steady-State Single Nucleotide Kinetic Parameters of Thermococcus sp. 9°N polD<sup>a,b</sup>

| dNTP     | \( k_{pol} (s^{-1}) \) | \( K_d (\mu M) \) | \( k_{pol}/K_d (\mu M^{-1} s^{-1}) \) | Nucleotide Selectivity<sup>c</sup> |
|----------|-------------------------|-------------------|--------------------------------------|----------------------------------|
| Template A |                         |                   |                                      |                                  |
| dTTP     | 1.8 ± 0.1               | 1.6 ± 0.1         | 1.1                                  | -                                |
| dATP     | 0.15 ± 0.01             | 390 ± 70          | 3.9 × 10⁻⁴                           | 2.8 × 10<sup>3</sup>             |
| dCTP     | 0.22 ± 0.02             | 450 ± 30          | 4.9 × 10⁻⁴                           | 2.2 × 10<sup>3</sup>             |
| dGTP     | 0.18 ± 0.01             | 330 ± 80          | 5.5 × 10⁻⁴                           | 2.0 × 10<sup>3</sup>             |
| Template G |                         |                   |                                      |                                  |
| dCTP     | 3.1 ± 0.8               | 1.7 ± 0.1         | 1.8                                  | -                                |
| dATP     | 0.12 ± 0.01             | 320 ± 70          | 3.8 × 10⁻⁴                           | 4.7 × 10<sup>3</sup>             |
| dTTP     | 0.38 ± 0.04             | 650 ± 150         | 5.9 × 10⁻⁴                           | 3.0 × 10<sup>3</sup>             |
| dGTP     | 0.07 ± 0.01             | 320 ± 80          | 2.2 × 10⁻⁴                           | 8.1 × 10<sup>3</sup>             |
| Template C |                         |                   |                                      |                                  |
| dGTP     | 2.1 ± 0.1               | 0.9 ± 0.3         | 2.3                                  | -                                |
| dATP     | 1.3 ± 0.1               | 570 ± 140         | 2.3 × 10⁻³                           | 1.0 × 10<sup>3</sup>             |
| dTTP     | 0.55 ± 0.09             | 1400 ± 500        | 3.9 × 10⁻⁴                           | 5.9 × 10<sup>3</sup>             |
| dCTP     | 0.42 ± 0.02             | 390 ± 120         | 1.0 × 10⁻³                           | 2.3 × 10<sup>3</sup>             |
| Template T |                         |                   |                                      |                                  |
| dATP     | 2.6 ± 0.7               | 2.5 ± 0.1         | 1.0                                  | -                                |
| dTTP     | 0.28 ± 0.02             | 490 ± 80          | 5.7 × 10⁻⁴                           | 1.8 × 10<sup>3</sup>             |
| dCTP     | 0.35 ± 0.03             | 530 ± 90          | 6.6 × 10⁻⁵                           | 1.5 × 10<sup>3</sup>             |
| dGTP     | 0.11 ± 0.01             | 300 ± 60          | 3.6 × 10⁻⁴                           | 2.8 × 10<sup>3</sup>             |

<sup>a</sup>Correctly incorporated nucleotides in bold
<sup>b</sup>Independent experiments were performed at least twice to ensure reproducibility. Reported values are from a typical experiment and error is derived from fit to the hyperbolic equation.
<sup>c</sup>Calculated as \( (k_{pol}/K_d)_{correct}/(k_{pol}/K_d)_{incorrect} \)

Table 4. Comparison of Ribonucleotide Incorporation Kinetics for Polymerase Families A, B, D, Y and RT

| Polymerase | Family | \( k_{pol} (s^{-1}) \) | \( K_d (\mu M) \) | \( k_{pol}/K_d (\mu M^{-1} s^{-1}) \) | Selectivity |
|------------|--------|-------------------------|-------------------|--------------------------------------|-------------|
| KF<sup>1</sup> | A      | (4.7 ± 2.5) × 10⁻²     | 21 ± 7            | 2.3 × 10⁻³                           | 3.4 × 10⁴   |
| RB69<sup>1</sup> | B      | 0.74 ± 0.2              | (1.6 ± 0.4) × 10⁻⁴| 4.6 × 10⁻⁵                           | 6.4 × 10⁴   |
| 9°N polD<sup>5</sup> | D      | 0.16 ± 0.01             | 360 ± 60          | 4.4 × 10⁻³                           | 2.3 × 10³   |
| Dbh<sup>6</sup> | Y      | (1.4 ± 0.3) × 10⁻⁵     | 770 ± 360         | 1.8 × 10⁻⁸                           | 3.4 × 10³   |
| HIV-1<sup>7</sup> | RT     | .03                     | 820 ± 150         | 3.7 × 10⁻⁵                           | 1.3 × 10⁵   |

<sup>1</sup>(45), <sup>2</sup>(24), <sup>3</sup>This work, <sup>4</sup>(47), <sup>5</sup>(49)
Table 5. Comparison of Pre-steady-state Nucleotide Incorporation Kinetics for Polymerase Families A, B, C, D, X, Y and RT

| Polymerase        | $k_{pol}$ (s$^{-1}$) | $K_d$ (μM) | $k_{pol}/K_d$ (μM$^{-1}$ s$^{-1}$) |
|-------------------|-----------------------|------------|----------------------------------|
| **Family A**      |                       |            |                                  |
| KF$^a$            | 50                    | 5.5        | 9.0                              |
| T7$^b$            | 120                   | 2          | 60                               |
| Klentaq$^c$       | 21                    | 35         | 0.60                             |
| **Family B**      |                       |            |                                  |
| Vent polB$^d$     | 66                    | 70         | 0.95                             |
| RB69$^e$          | 200                   | 69         | 2.9                              |
| T4$^f$            | >400                  | 20         | 20                               |
| human pol ε$^g$   | 248                   | 31         | 8                                |
| yeast pol δ$^h$   | 0.93                  | 24         | 0.04                             |
| **Family C**      |                       |            |                                  |
| Sau PolC$^i$      | 180                   | 4          | 45                               |
| **Family D**      |                       |            |                                  |
| 9°N PolD$^j$      | 3.1                   | 1.7        | 1.8                              |
| **Family X**      |                       |            |                                  |
| rPolβ$^k$         | 12.5                  | 1.9        | 6.6                              |
| **Family Y**      |                       |            |                                  |
| Dpo4$^l$          | 7.6                   | 70         | 0.10                             |
| **Reverse transcriptase** |             |            |                                  |
| HIV-1$^m$         | 26                    | 9          | 2.88                             |

$^a$(21), $^b$(50), $^c$(51), $^d$(25), $^e$(52), $^f$(53), $^g$(27), $^h$(54), $^i$(55), $^j$(23), $^k$(40), $^l$(56)
### Table 6. Comparison of the Average Nucleotide Selectivity for Polymerase Families A, B, D, X and Y

| Polymerase | Family | \(k_{pol}/K_d\) correct (µM⁻¹ s⁻¹) | \(k_{pol}/K_d\) incorrect (µM⁻¹ s⁻¹) | Nucleotide Selectivity\(^a\) |
|------------|--------|---------------------------------|---------------------------------|--------------------------|
| KF\(^b\)  | A      | 11                              | \(1.2 \times 10^{-3}\)          | \(4.1 \times 10^{4}\)   |
| RB69\(^c\) | B      | 2.9                             | \(1.1 \times 10^{-3}\)          | \(2.7 \times 10^{5}\)   |
| 9°N PolD\(^d\) | D      | 1.6                             | \(6.1 \times 10^{-4}\)          | \(4.3 \times 10^{4}\)   |
| rPolβ\(^e\) | X      | 5.0                             | \(5.9 \times 10^{-4}\)          | \(3.3 \times 10^{4}\)   |
| Dpo4\(^f\) | Y      | \(7.0 \times 10^{-2}\)          | \(3.3 \times 10^{-3}\)          | \(3.0 \times 10^{3}\)   |

\(^a\)Calculated from \((k_{pol}/K_d)\)correct/\((k_{pol}/K_d)\)incorrect, \(^b\)(39), \(^c\)This work, \(^d\)(23), \(^e\)(40)
FIGURES

Figure 1.
Figure 2.
Figure 3.
Figure 4.

A. Kinetics of a Family D DNA polymerase

B. Time course of 5'-FAM labeled 50 nt DNA fragment with polD- nuclease:
   I. 0 sec
   II. 0.5 sec
   III 1.0 sec
   IV. 2.0 sec
   V. 5.0 sec
   VI. 10 sec
   VII. 15 sec
   VIII. 30 sec

C. Graph showing observed rate constant ($k_{obs}$) vs. [PPi] concentration.

$\text{obs} (\text{s}^{-1})$

$\text{[PPi] } \mu\text{M}$
Figure 5.

A.

B.

I. 0 sec
II. 0.002 sec
III. 0.004 sec
IV. 0.008 sec
V. 0.075 sec
VI. 0.25 sec

I. 0 sec
II. 0.075 sec
III. 0.1 sec
IV. 0.25 sec
V. 0.5 sec
VI. 1.0 sec

C.
Figure 6.

**Polymerization**

\[
\begin{align*}
E\cdot DNA_n + dNTP & \rightarrow E\cdot DNA_n \cdot dNTP \\
& \xrightarrow{\text{"open"}} E\cdot DNA_n \cdot dNTP \\
& \xrightarrow{\text{"closed"}} E\cdot DNA_n \cdot dNTP \\
& \xrightarrow{k_{\text{pol}}} E\cdot DNA_{n+1} \cdot PPI \\
& \xrightarrow{k_{\text{pyro}}} E\cdot DNA_{n+1} \cdot PPI \\
& \xrightarrow{k_{\text{PPI}}} E\cdot DNA_{n+1} \\
& \xrightarrow{k_{\text{dPPI}}} E + DNA_{n+1}
\end{align*}
\]

- \( K_d \cdot dNTP \approx 1.7 \mu M \\
- k_{\text{pol}} \approx 2.5 s^{-1} \\
- k_{\text{pyro}} \approx 0.4 s^{-1} \\
- k_{\text{PPI}} \approx 0.3 s^{-1} \\
- K_d \cdot PPI = 190 \mu M

**3'-5' Exonuclease ssDNA Hydrolysis**

\[
\begin{align*}
E + ssDNA_n & \equiv E + ssDNA_n \\
& \xrightarrow{k_{\text{exo}}} E + ssDNA_{n-1} + dNMP \\
& \xrightarrow{k_{\text{extra}}} E + ssDNA_{n-1}
\end{align*}
\]

- \( Mg^{2+} \approx 2.5 s^{-1} \\
- Mn^{2+} \approx 110 s^{-1} \]