Research Article

Pre-Steady-State Kinetic Analysis of Truncated and Full-Length Saccharomyces cerevisiae DNA Polymerase Eta

Jessica A. Brown,¹ Likui Zhang,¹ Shanen M. Sherrer,¹ John-Stephen Taylor,² Peter M. J. Burgers,³ and Zucai Suo¹

¹Department of Biochemistry, The Ohio State University, Columbus, OH 43210, USA

² Department of Chemistry, Washington University, St. Louis, MO 63130, USA

³ Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, USA

Correspondence should be addressed to Zucai Suo, suo.3@osu.edu

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Understanding polymerase fidelity is an important objective towards ascertaining the overall stability of an organism's genome. *Saccharomyces cerevisiae* DNA polymerase η (yPol η), a Y-family DNA polymerase, is known to efficiently bypass DNA lesions (e.g., pyrimidine dimers) in vivo. Using pre-steady-state kinetic methods, we examined both full-length and a truncated version of yPol η which contains only the polymerase domain. In the absence of yPol η 's C-terminal residues 514–632, the DNA binding affinity was weakened by 2-fold and the base substitution fidelity dropped by 3-fold. Thus, the C-terminus of yPol η may interact with DNA and slightly alter the conformation of the polymerase domain during catalysis. In general, yPol η discriminated between a correct and incorrect nucleotide more during the incorporation step (50-fold on average) than the ground-state binding step (18-fold on average). Blunt-end additions of dATP or pyrene nucleotide 5'-triphosphate revealed the importance of base stacking during the binding of incorrect incoming nucleotides.

1. Introduction

DNA polymerases are organized into seven families: A, B, C, D, X, Y, and reverse transcriptase [1, 2]. Among these families, DNA polymerases are involved in DNA replication, DNA repair, DNA lesion bypass, antibody generation, and sister chromatid cohesion [3]. Despite these diverse roles, DNA polymerases catalyze the nucleotidyl transfer reaction using a two divalent metal ion mechanism [4] with at least one positively charged residue [5] that functions as a general acid [6] at their active site, follow a similar minimal kinetic pathway [7], and share a similar structural architecture consisting of the fingers, palm, and thumb subdomains [8, 9]. Surprisingly, the polymerization fidelity of eukaryotic DNA polymerases spans a wide range: one error per one to one billion nucleotide incorporations (10^0 to 10^{-9}) [10].

The Y-family DNA polymerases are known for catalyzing nucleotide incorporation with low fidelity and poor processivity. These enzymes are specialized for translesion DNA synthesis which involves nucleotide incorporation opposite and downstream of a damaged DNA site. Lesion bypass can be either error-free or error-prone depending on the DNA polymerase and DNA lesion combination. To accommodate a distorted DNA substrate, Y-family DNA polymerases utilize several features: a solvent-accessible [11] and conformationally flexible active site [12], smaller fingers and thumb subdomains [11], an additional subdomain known as the little finger [11], the little finger and polymerase core domains move in opposite directions during a catalytic cycle [13], and a lack of $3' \rightarrow 5'$ exonuclease activity [14]. Unfortunately, these features, which facilitate lesion bypass, may also contribute to the low fidelity of a Y-family DNA polymerase during replication of a damaged or undamaged



FIGURE 1: Schematic illustration of yPol η . The polymerase domain of yPol η is at the N-terminus while a ubiquitin-binding zinc finger (UBZ) domain and PCNA-interacting peptide (PIP) motif is at the C-terminus. Residue numbers are denoted above each region. For this study, the truncated construct contains only the polymerase domain.

DNA template. Thus, it is important to understand the mechanism and fidelity of the Y-family DNA polymerases.

Saccharomyces cerevisiae DNA polymerase η (yPol η), a Yfamily DNA polymerase, is critical for the error-free bypass of UV-induced DNA damage such as a cis-syn thyminethymine dimer [15–19]. To date, Pol η remains the only Y-family DNA polymerase with a confirmed biological function [20]. vPoln is organized into a polymerase domain, ubiquitin-binding zinc finger (UBZ) domain, and proliferating cell nuclear antigen- (PCNA) interacting peptide (PIP) motif (Figure 1). X-ray crystal structures of $vPol\eta$'s catalytic core have been solved alone [21] as well as in complex with a cisplatin-DNA adduct and an incoming nucleotide [22]. Due to a lack of structures for full-length yPol η , it is unclear if the C-terminal residues 514-632 interact with DNA and contribute to the polymerase function of yPol_η. Using pre-steady-state kinetic techniques, we have measured the base-substitution fidelity of full-length and truncated yPol η (Figure 1) catalyzing nucleotide incorporation into undamaged DNA. In addition, we have determined the DNA binding affinity of both full-length and truncated yPoln. Our results show that the C-terminus of yPol η has a minor effect on the DNA binding affinity and the base substitution fidelity of this lesion bypass DNA polymerase.

2. Materials and Methods

2.1. *Materials*. Materials were purchased from the following companies: $[\gamma^{-32}P]$ ATP, MP Biomedicals (Solon, OH); Biospin columns, Bio-Rad Laboratories (Herclues, CA); dNTPs, GE Healthcare (Piscataway, NJ); oligodeoxyribonucleotides, Integrated DNA Technologies, Inc. (Coralville, IA); and OptiKinase, USB (Cleveland, OH).

2.2. Preparation of Substrates and Enzymes. The synthetic oligodeoxyribonucleotides listed in Table 1 were purified as described previously [23]. The primer strand 21-mer or blunt-end 16-mer was 5'-radiolabeled with $[\gamma^{-32}P]$ ATP and OptiKinase. Then, the 21-mer was annealed to the appropriate 41 mer template (Table 1) and the palindromic blunt-end substrates were annealed as described previously [23]. The catalytic core of yPol η (1–513) containing an N-terminal

TABLE 1: Sequences of DNA substrates^a.

D-1	5'-CGCAGCCGTCCAACCAACTCA-3' 3'-GCGTCGGCAGGTTGGTTGAGTAGCAGCTAGGTTACGGCAGG-5'
D-6	5'-CGCAGCCGTCCAACCAACTCA-3' 3'-GCGTCGGCAGGTTGGTTGAGTGGCAGGTACGGCAGG-5
D-7	5'-CGCAGCCGTCCAACCAACTCA-3' 3'-GCGTCGGCAGGTTGGTTGAGTTGCAGCTAGGTTACGGCAGG-5'
D-8	5'-CGCAGCCGTCCAACCAACTCA-3' 3'-GCGTCGGCAGGTTGGTTGAGTCGCAGCTAGGTTACGGCAGG-5'
F-8	5'-CGCAGCCGTCCAACCAACTCA-3' 3'-GCGTCGGCAGGTTGGTTGAGTCXCAGCTAGGTTACGGCAGG-5'
BE1	5'-ATGAGTTGCAACTCAT-3' 3'-TACTCAACGTTGAGTA-5'
BE2	5'-TTGAGTTGCAACTCAA-3' 3'-AACTCAACGTTGAGTT-5'
BE3	5'-CTGAGTTGCAACTCAG-3' 3'-GACTCAACGTTGAGTC-5'
BE4	5'-GTGAGTTGCAACTCAC-3' 3'-CACTCAACGTTGAGTG-5'

^aThe template base highlighted in bold is unique to each strand and **X** denotes 2-aminopurine.

MGSSH₆SSGLVPRGSH tag was purified as described previously [24]. The full-length yPol η (1–632) was expressed and purified from yeast [25]. Pyrene 5'-triphosphate (dPTP) was synthesized as described previously [26].

2.3. Pre-Steady-State Kinetic Assays. All experiments were performed in reaction buffer A which contained 40 mM Tris-HCl pH 7.5 at 23°C, 5 mM MgCl₂, 1 mM DTT, 10 µg/mL BSA, and 10% glycerol. A rapid chemical-quench flow apparatus (KinTek, PA, USA) was used for fast reactions. For burst assays, a preincubated solution of $yPol\eta$ (320 nM) and 5'-[32P]-labeled D-1 DNA (480 nM) was mixed with dTTP·Mg²⁺ (100 μ M). To measure the dissociation rate of the yPol η ·DNA binary complex, a preincubated solution of yPol η (50 nM) and 5'-[³²P]-labeled D-1 DNA (100 nM) was mixed with a molar excess of unlabeled D-1 DNA $(2.5 \,\mu\text{M})$ for various time intervals prior to initiating the polymerization reaction with dTTP·Mg²⁺ (150 and 400 μ M for truncated and full-length yPol η , resp.) for 15 s. For singleturnover kinetic assays, a preincubated solution of $yPol\eta$ (150 nM) and 5'-[³²P]-labeled DNA (30 nM) was mixed with an incoming $dNTP \cdot Mg^{2+}$ (0.4–800 μM). Reactions were quenched at the designated time by adding 0.37 M EDTA. Reaction products were analyzed by sequencing gel electrophoresis (17% acrylamide, 8 M urea, 1 × TBE running buffer), visualized using a Typhoon TRIO (GE Healthcare), and quantitated with ImageQuant software (Molecular Dynamics).

2.4. DNA Binding Assays. The equilibrium dissociation constant (K_d^{DNA}) of the yPol η ·DNA binary complex was determined using two techniques. First, an electrophoretic mobility shift assay (EMSA) was employed by adding increasing concentrations of yPol η (10–450 nM) into a fixed concentration of 5'-[³²P]-labeled D-1 DNA (10 nM) in

buffer A. The solution established equilibrium during a 20minute incubation period. Then, the binary complex was separated from unbound DNA using a 4.5% native polyacrylamide gel and running buffer as previously described except the final concentration of Tris was adjusted to 40 mM [27]. Second, a fluorescence titration assay was used. Increasing concentrations of yPol η (2–300 nM) were titrated into a fixed concentration of F-8 DNA (25 nM) in buffer A (devoid of BSA). The F-8 DNA substrate (Table 1) was excited at a wavelength of 312 nm with emission and excitation slit widths of 5 nm. The emission spectra were collected at 1 nm intervals from 320 to 500 nm using a Fluoromax-4 (Jobin Jvon Horiba). Emission background from the buffer and intrinsic protein fluorescence were subtracted from each spectrum.

2.5. Data Analysis. For the pre-steady-state burst assay, the product concentration was graphed as a function of time (t) and the data were fit to the burst equation (1) using the nonlinear regression program, KaleidaGraph (Synergy Software):

$$[Product] = A[1 - \exp(-k_1t) + k_2t].$$
(1)

A represents the fraction of active enzyme, k_1 represents the observed burst rate constant, and k_2 represents the observed steady-state rate constant.

Data for the EMSA were graphed by plotting the concentration of the binary complex as a function of enzyme concentration (E_0) and fitting it to a quadratic equation (2):

$$[\mathbf{E} \cdot \mathbf{DNA}] = 0.5 \left(K_d^{\mathbf{DNA}} + \mathbf{E}_0 + \mathbf{D}_0 \right)$$
$$- 0.5 \left[\left(K_d^{\mathbf{DNA}} + \mathbf{E}_0 + \mathbf{D}_0 \right)^2 - 4\mathbf{E}_0 \mathbf{D}_0 \right]^{1/2}.$$
(2)

 \mathbf{D}_0 is the DNA concentration.

For the fluorescence titration experiments, a modified quadratic equation (3) was applied to a plot of the fluorescence intensity (F) measured at 370 nm versus enzyme concentration:

$$[F] = F_{max} + \left[\frac{(F_{min} - F_{max})}{2D_0}\right] \times \left\{ \left(K_d^{DNA} + E_0 + D_0\right) - \left[\left(K_d^{DNA} + E_0 + D_0\right)^2 - 4E_0D_0\right]^{1/2} \right\}.$$
(3)

 F_{max} and F_{min} represent the maximum and minimum fluorescence intensity, respectively.

For the rate of DNA dissociation from the binary complex, a single-exponential equation (4) was applied to a plot of product concentration versus time:

$$[Product] = A[exp(-k_{off}t)] + C.$$
(4)

A represents the reaction amplitude, k_{off} is the observed rate constant of DNA dissociation, and C is the concentration of

the radiolabeled DNA product in the presence of a DNA trap for unlimited time.

For the single-turnover kinetic assays, a plot of product concentration versus time was fit to a single-exponential equation (5) to extract the observed rate constant of nucleotide incorporation (k_{obs}):

$$[Product] = A[1 - \exp(-k_{obs}t)].$$
(5)

To measure the maximum rate constant of incorporation (k_p) and the apparent equilibrium dissociation constant (K_d) of an incoming nucleotide, the extracted k_{obs} values were plotted as a function of nucleotide concentration and fit to a hyperbolic equation (6):

$$[k_{\rm obs}] = \frac{k_p [\rm dNTP]}{(K_d + [\rm dNTP])}.$$
(6)

The free energy change ($\Delta\Delta G$) for a correct and incorrect nucleotide substrate dissociating from the E·DNA·dNTP complex was calculated according to (7).

$$\Delta\Delta G = RT \ln \left[\frac{(K_d)_{\text{incorrect}}}{(K_d)_{\text{correct}}} \right].$$
(7)

Here, R is the universal gas constant and T is the reaction temperature in Kelvin.

3. Results and Discussion

3.1. Truncated and Full-Length yPoln Display Biphasic Kinetics. Previously, transient state kinetic techniques have been used to characterize full-length yPol η at 30°C [28]. Therefore, we first performed a burst assay (see Section 2) to ensure that our purified proteins, truncated and full-length yPol η (Figure 1), behaved in a similar manner at 23°C. Compared to wild-type yPol η , the truncated construct contains only the polymerase domain (Figure 1). A preincubated solution of yPol_{\eta} (320 nM) and 5'-[³²P]-labeled 21/41 mer D-1 DNA (480 nM) was mixed with dTTP·Mg²⁺ (100 μ M) and quenched with EDTA at various times. Product concentration was plotted as a function of time and was fit to (1), since there were two distinct kinetic phases: a rapid, exponential phase and a slow, linear phase (data not shown). These burst results were similar to those previously published [28]. Biphasic kinetics of nucleotide incorporation indicated that the first turnover rate was the rate of nucleotide incorporation occurring at the enzyme's active site while subsequent turnovers (i.e., linear phase) were likely limited by the DNA product release step as demonstrated by fulllength yPol η at 30°C [28] and other DNA polymerases [23, 29, 30].

3.2. The C-Terminal 119 Residues Slightly Enhance DNA Binding Affinity of yPol η . The equilibrium dissociation constant for the binary complex of yPol η ·DNA (K_d^{DNA}) was measured to determine if the C-terminus of yPol η affects DNA binding affinity (Scheme 1). First, the K_d^{DNA} was estimated using the EMSA (see Section 2). For example, varying concentrations





of full-length yPol η (10–450 nM) were incubated with a fixed concentration of 5'-[³²P]-labeled D-1 DNA (10 nM) before separating the binary complex from the unbound DNA on a native gel (Figure 2(a)). Then, a quadratic equation (2) was applied to a plot of the binary complex concentration versus yPol η concentration which resolved a K_d^{DNA} of 16 ± 1 nM (Figure 2(b) and Table 2). Under similar reaction conditions, the K_d^{DNA} of truncated yPol η was estimated to be 34 ± 3 nM, a binding affinity ($1/K_d^{\text{DNA}}$) value that is 2-fold weaker than that of full-length yPol η (Table 2).

To corroborate these estimated K_d^{DNA} values, we measured the true K_d^{DNA} for the yPol $\eta \cdot D$ NA complex using a fluorescence titration assay. An analog of dA, 2-aminopurine, was embedded into the 41 mer template of F-8 DNA which is identical to 21/41 mer D-8 DNA except that 2-aminopurine flanks the 5' end of the templating dC base (Table 1). The F-8 DNA substrate (25 nM) was excited at 312 nm, and the emission spectrum was collected from 320 to 500 nm. After serial additions of full-length or truncated $yPol\eta$ in independent titrations, a decrease in the fluorescence intensity of F-8 was observed. These changes in fluorescence intensity at 370 nm were plotted as a function of the yPol η concentration and were fit to (3) to extract a K_d^{DNA} equal to $7 \pm 4 \text{ nM}$ for full-length yPol η (Figure 2(c)) and $13 \pm 5 \text{ nM}$ for truncated yPol η (Table 2). These K_d^{DNA} measurements were tighter than those determined using EMSA, since the fluorescence titration assay allows yPol η to associate and dissociate during data collection. In contrast, EMSA does not maintain a constant equilibrium because dissociated yPoln cannot reassociate with DNA during electrophoresis separation. Nonetheless, there was a confirmed ~2-fold difference in the DNA binding affinity between full-length and the catalytic core of yPol η which indicates that the Cterminal 119 amino acid residues of yPol η slightly enhance the binding of the enzyme to DNA.

Next, we directly measured the rate of DNA dissociation from the yPol η ·DNA complex (see Section 2). A preincubated solution of yPol η (50 nM) and 5'-radiolabeled D-1 DNA (100 nM) was combined with a 50-fold molar excess of unlabeled D-1 DNA for various time intervals before dTTP was added for 15 s to allow ample extension of the labeled D-1 DNA that remained in complex with yPol η . A plot of product concentration versus the incubation time with the unlabeled DNA trap (data not shown) was fit to (4) which yielded DNA dissociation rates (k_{off}) of 0.008 ± 0.001 s⁻¹ and 0.0041 ± 0.0008 s⁻¹ for truncated

TABLE 2: Rate and equilibrium dissociation constants for the binary complex yPol η ·DNA at 23°C.

Kinetic Parameter	Truncated yPol η	Full-length yPol η
$k_{\rm on} \; (\mu {\rm M}^{-1} \; {\rm s}^{-1})^{\rm a}$	0.62	0.59
$k_{\rm off} ({ m s}^{-1})$	0.008 ± 0.001	0.0041 ± 0.0008
$K_d^{\mathrm{DNA}} \left(\mathrm{nM}\right)^{\mathrm{b}}$	34 ± 3	16 ± 1
$K_d^{\mathrm{DNA}} (\mathrm{nM})^{\mathrm{c}}$	13 ± 5	7 ± 4

^aCalculated as $k_{\rm off}/K_d^{\rm DNA}$. The $K_d^{\rm DNA}$ value was measured from a fluorescence titration assay.

^bEstimated using EMSA.

^cMeasured using a fluorescence titration assay.

and full-length yPol η , respectively (Table 2 and Scheme 1). Interestingly, the rate of DNA dissociation from full-length yPol η is 2-fold slower than that from truncated yPol η , which indicated that the C-terminus of yPol η may slightly contribute to this polymerase's DNA binding affinity.

Based on the measured K_d^{DNA} from Figure 2(c) and k_{off} values, the apparent second-order association rate constant $(k_{\text{on}} = k_{\text{off}}/K_d^{\text{DNA}})$ of the binary complex $y\text{Pol}\eta\cdot\text{DNA}$ was calculated to be 0.62 and $0.59\,\mu\text{M}^{-1}\,\text{s}^{-1}$ for truncated and full-length $y\text{Pol}\eta$, respectively (Table 2). These similar k_{on} values indicate that the slightly stronger DNA binding affinity of full-length $y\text{Pol}\eta$ is mainly due to a slightly slower rate of DNA dissociation (k_{off}) . Taken together, the data in Table 2 suggest that the C-terminal 119 amino acid residues of $y\text{Pol}\eta$ slightly hinder the dissociation of DNA from the binary complex $y\text{Pol}\eta\cdot\text{DNA}$. This hindrance is through either direct physical interactions between the C-terminus of $y\text{Pol}\eta$ and DNA, modulation of the conformation of the polymerase domain by the C-terminus of $y\text{Pol}\eta$, or both.

3.3. Base Substitution Fidelity of Truncated yPol η . Since a presteady-state burst was observed for truncated yPol η , we continued to investigate the nucleotide incorporation efficiency (k_p/K_d) by measuring the maximum rate of nucleotide incorporation (k_p) and the apparent equilibrium dissociation constant (K_d) of an incoming nucleotide under single-turnover conditions [31]. By performing these experiments with yPol η in molar excess over DNA, the conversion of D-DNA_n to D-DNA_{n+1} (Scheme 1) was directly observed in a single pass through the enzymatic pathway [32]. A preincubated solution of truncated yPol η (150 nM) and 5'-[³²P]-labeled D-7 DNA (30 nM) was mixed with varying concentrations of dATP·Mg²⁺ (0.4–80 μ M) and quenched with EDTA at



FIGURE 2: Equilibrium dissociation constant for full-length yPol η . (a) Gel image showing binary complex formation at various concentrations of full-length yPol η (10–450 nM) in the presence of 5'-[³²P]-labeled D-1 DNA (10 nM). (b) The concentration of the binary complex was plotted as a function of full-length yPol η concentration and fit to (2) to yield a $K_d^{\text{DNA}} = 16 \pm 1$ nM. (c) For the fluorescence titration assay, a plot of fluorescence intensity versus full-length yPol η concentration was fit to (3) which resolved a $K_d^{\text{DNA}} = 7 \pm 4$ nM.

various times (see Section 2). A plot of product concentration versus time was fit to (5) to extract the observed rate constant (k_{obs}) for dATP incorporation (Figure 3(a)). Then, the k_{obs} values were plotted as a function of dATP concentration and fit to a hyperbolic equation (6) which resolved a k_p of $6.9 \pm 0.4 \, \text{s}^{-1}$ and an apparent K_d of $17 \pm 3 \, \mu$ M (Figure 3(b)). The pre-steady-state kinetic parameters for the remaining 15 possible dNTP:dN base pair combinations were determined under single-turnover conditions and were used to calculate the substrate specificity constant (k_p/K_d), discrimination factor ($(k_p/K_d)_{correct}/(k_p/K_d)_{incorrect}$]) of truncated yPol η (Table 3).

Overall, the base substitution fidelity of truncated yPol η was in the range of 10^{-2} to 10^{-4} which translates into 1 misincorporation per 100 to 10,000 nucleotide incorporations (Table 3). Depending on the mispair, truncated yPol η catalyzed a misincorporation with 30- to 2,700-fold (640-fold on average) lower efficiency than the corresponding correct base pair. To better understand the mechanistic basis of truncated yPol η 's fidelity, the equation for polymerase fidelity can be simplified as follows:

Fidelity =
$$\frac{\left(k_{p}/K_{d}\right)_{\text{incorrect}}}{\left[\left(k_{p}/K_{d}\right)_{\text{correct}} + \left(k_{p}/K_{d}\right)_{\text{incorrect}}\right]}$$
$$\approx \frac{\left(k_{p}/K_{d}\right)_{\text{incorrect}}}{\left(k_{p}/K_{d}\right)_{\text{correct}}}$$
$$= \left[\frac{\left(k_{p}\right)_{\text{incorrect}}}{\left(k_{p}\right)_{\text{correct}}}\right]^{-1} \left[\frac{\left(K_{d}\right)_{\text{correct}}}{\left(K_{d}\right)_{\text{incorrect}}}\right]^{-1}$$
$$= (\text{rate difference})^{-1} (\text{binding affinity difference})^{-1}.$$
(8)

Thus, fidelity is inversely proportional to the rate difference and apparent binding affinity difference between correct and incorrect nucleotide incorporation. In general, the mechanistic basis of $yPol\eta$'s discrimination was due to a 3- to 68-fold (18-fold on average) weaker apparent binding affinity ($1/K_d$) and 5- to 220-fold (50-fold on average) slower rate constant of incorporation for a mismatched dNTP.

3.4. Kinetic Significance of Base Stacking Contributing to the Binding Affinity of an Incoming Nucleotide. Although all four correct dNTPs were bound with similarly high affinity (Table 3), mismatched purine deoxyribonucleotides have 2-to 6-fold lower apparent K_d values than mismatched pyrimidine deoxyribonucleotides. Because 5'-protruding purines have been found to have stronger stacking interactions with a terminal DNA base pair than 5'-protruding pyrimidines [33], the difference in apparent K_d values suggests that base-stacking interactions between an incorrect dNTP and the terminal primer/template base pair dA:dT (Table 1) play a role on the binding of dNTP by truncated yPol η . Interestingly, we have previously demonstrated that the preferred nucleotide for template-independent nucleotide



FIGURE 3: Concentration dependence on the pre-steady-state rate constant of nucleotide incorporation catalyzed by truncated yPol η . (a) A preincubated solution of truncated yPol η (150 nM) and 5'-[³²P]-labeled D-7 DNA (30 nM) was mixed with dATP·Mg²⁺ (0.4 μ M, \oplus ; 0.8 μ M, \bigcirc ; 2 μ M, \blacksquare ; 4 μ M, \Box ; 8 μ M, \blacktriangle ; 16 μ M, \triangle ; 40 μ M, \blacklozenge ; 80 μ M, \diamondsuit) and quenched with EDTA at various time intervals. The solid lines are the best fits to a single-exponential equation which determined the observed rate constant, k_{obs} . (b) The k_{obs} values were plotted as a function of dATP concentration. The data ($\textcircled{\bullet}$) were then fit to a hyperbolic equation, yielding a k_p of 6.9 ± 0.4 s⁻¹ and a K_d of 17 ± 3 μ M.

TABLE 3: Kinetic parameter	s of nucleotide incor	poration into D-DNA	catalyzed b	y truncated y	yPolr	1 at 23°	°C.
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dNTP	$k_p (s^{-1})$	$K_d \; (\mu \mathrm{M})$	$k_p/K_d(\mu M^{-1}s^{-1})$	Discrimination Factor ^a	Fidelity ^b
Template a	lA (D-1)				
dTTP	3.9 ± 0.2	15 ± 2	$2.6 imes 10^{-1}$		
dATP	0.089 ± 0.005	80 ± 20	$1.1 imes 10^{-3}$	230	$4.3 imes 10^{-3}$
dCTP	0.43 ± 0.06	210 ± 60	$2.0 imes10^{-3}$	130	$7.8 imes10^{-3}$
dGTP	0.15 ± 0.01	80 ± 20	$1.9 imes10^{-3}$	140	$7.2 imes 10^{-3}$
Template a	lG (D-6)				
dCTP	15.6 ± 0.3	11.2 ± 0.8	1.4		
dATP	0.071 ± 0.002	138 ± 9	$5.1 imes10^{-4}$	2700	$3.7 imes10^{-4}$
dGTP	0.116 ± 0.006	80 ± 10	$1.5 imes10^{-3}$	960	$1.0 imes10^{-3}$
dTTP	0.92 ± 0.07	330 ± 40	$2.8 imes 10^{-3}$	500	$2.0 imes10^{-3}$
Template a	lT (D-7)				
dATP	6.9 ± 0.4	17 ± 3	$4.1 imes 10^{-1}$		
dCTP	1.00 ± 0.04	210 ± 20	$4.8 imes 10^{-3}$	85	$1.2 imes 10^{-2}$
dGTP	0.55 ± 0.01	46 ± 3	$1.2 imes 10^{-2}$	30	$2.9 imes 10^{-2}$
dTTP	0.62 ± 0.02	280 ± 20	2.2×10^{-3}	180	$5.4 imes 10^{-3}$
Template a	lC (D-8)				
dGTP	6.3 ± 0.1	6.8 ± 0.4	$9.3 imes10^{-1}$		
dATP	0.087 ± 0.003	90 ± 10	$9.7 imes10^{-4}$	960	$1.0 imes10^{-3}$
dCTP	0.127 ± 0.007	200 ± 30	$6.4 imes10^{-4}$	1500	$6.9 imes10^{-4}$
dTTP	1.39 ± 0.06	460 ± 40	$3.0 imes 10^{-3}$	310	3.3×10^{-3}

^aCalculated as $(k_p/K_d)_{\text{correct}}/(k_p/K_d)_{\text{incorrect}}$.

^bCalculated as $(k_p/K_d)_{\text{incorrect}}/[(k_p/K_d)_{\text{correct}} + (k_p/K_d)_{\text{incorrect}}].$

DNA (Terminal base pair)	dNTP	$k_{p} (s^{-1})$	$K_d \ (\mu M)$	$k_p/K_d \; (\mu \mathrm{M}^{-1} \mathrm{s}^{-1})$	Efficiency Ratio ^a
$BE1(dT \cdot dA)$	dATP	0.026 ± 0.002	1200 ± 200	2.2×10^{-5}	_
DEI (dI.dA)	dPTP	1.27 ± 0.08	60 ± 10	$2.1 imes 10^{-2}$	980
PE2(dA, dT)	dATP	0.036 ± 0.002	220 ± 30	$1.6 imes10^{-4}$	_
DE2(ux.u1)	dPTP	0.68 ± 0.03	23 ± 3	$3.0 imes 10^{-2}$	180
PE2(AC, AC)	dATP	0.0087 ± 0.0003	360 ± 30	$2.4 imes 10^{-5}$	_
DES(uG:uC)	dPTP	0.22 ± 0.01	9 ± 2	$2.4 imes 10^{-2}$	1000
PE4(dC, dC)	dATP	0.032 ± 0.001	930 ± 70	$3.4 imes 10^{-5}$	_
DD4(uC.uG)	dPTP	0.74 ± 0.03	12 ± 2	$6.2 imes 10^{-2}$	1800

TABLE 4: Kinetic parameters for nucleotide incorporation onto blunt-end DNA catalyzed by truncated yeast Pol η at 23°C.

^aCalculated as $(k_p/K_d)_{dPTP}/(k_p/K_d)_{dATP}$.



FIGURE 4: Chemical structure of a nonnatural nucleotide analog, dPTP.

incorporation catalyzed by Dpo4, another Y-family DNA polymerase, is dATP mainly due to its strong intrahelical base-stacking ability [26]. To further evaluate the role of base stacking, we first examined if truncated yPol η can catalyze template-independent nucleotide incorporation of dATP or dPTP (Figure 4) onto four palindromic, bluntend DNA substrates (BE1, BE2, BE3, and BE4 in Table 1). The base of dPTP, a dNTP analog, has four conjugated benzene rings but possesses no hydrogen-bonding abilities. The DNA substrates possess all four possible terminal base pairs and each molecule of them can be bound by a single polymerase molecule. Our radioactive experiments showed that truncated yPol η was able to incorporate dATP and dPTP (data not shown). Then, we individually measured the kinetic parameters for dATP and dPTP incorporation under single-turnover reaction conditions (Table 4). Interestingly, the apparent K_d values of dATP were 3- to 5-fold smaller with a purine than those with a pyrimidine on the primer's 3'base, indicating that base stacking is also important for the binding of dATP to the binary complex of yPol η ·blunt-end DNA. This base-stacking effect is more dramatic for dPTP incorporation onto blunt-end DNA because the apparent K_d values of dPTP are 10- to 80-fold tighter than dATP incorporation onto the same blunt-end DNA substrate (Table 4). Thus, the binding free energy difference between dATP and dPTP is 1.4 to 2.6 kcal/mol. Previously, we have obtained a comparable binding free energy difference of 2.3 kcal/mol for similar blunt-end dATP and dPTP incorporation at 37°C

catalyzed by Dpo4 [26]. Although neither dATP nor dPTP forms any hydrogen bonds with a template base when bound by yPol η · blunt-end DNA, the bases of these two nucleotides should have different base-stacking interactions with a terminal base pair of a blunt-end DNA substrate considering that a dangling pyrene base (1.7 kcal/mol) has previously been found to possess a higher base-stacking free energy than a dangling adenosine (1.0 kcal/mol) [33]. However, the base-stacking free energy difference (0.7 kcal/mol) between pyrene and adenosine is smaller than the aforementioned binding free energy difference (1.4–2.6 kcal/mol) between dPTP and dATP. Thus, other sources likely contribute to the tighter binding of dPTP over dATP. One possible source is favorable van der Waals interactions between pyrene and active site residues of truncated yPoln. In addition, the basestacking effect and van der Waals interactions may stabilize the ternary complex of yPol η ·blunt-end DNA·nucleotide and facilitate catalysis, leading to much higher k_p values with dPTP than those with dATP (Table 4). Due to the differences in k_p and apparent K_d , the substrate specificity values of dPTP are 100- to 1,000-fold higher than those of dATP with blunt-end DNA (Table 4) and 10- to 100-fold higher than mismatched dATP with regular DNA (Table 3).

3.5. Base Substitution Fidelity of Full-Length yPoln. The base substitution fidelities of full-length and truncated yPol η may differ because the C-terminal, nonenzymatic regions may alter the polymerization fidelity. For example, the prolinerich domain of human DNA polymerase λ has been shown to upregulate the polymerase fidelity up to 100-fold [34]. To determine if the C-terminus of $yPol\eta$ influences polymerization fidelity, we measured the pre-steady-state kinetic parameters for dNTP incorporation into D-1 DNA (template dA) catalyzed by full-length yPol η (Table 5). The fidelity was calculated to be in the range of $(1.4 \text{ to } 2.6) \times 10^{-3}$ for fulllength yPol η (Table 5). Relative to the fidelity of truncated yPol η with D-1 (Table 3), full-length yPol η has a 3-fold higher fidelity. Therefore, the C-terminus of $yPol\eta$ slightly affects the base substitution fidelity. Moreover, truncated yPol η discriminated between a correct and incorrect dNTP by ~30-fold on average based on the k_p difference while the discrimination for full-length yPol η was ~170-fold on average for incorporation into D-1 DNA (Tables 3 and 5). The incorporation rate constant for correct dTTP was

dNTP	$k_{p} (s^{-1})$	$K_d \ (\mu M)$	$k_p/K_d \;(\mu \mathrm{M}^{-1}\mathrm{s}^{-1})$	Discrimination Factor ^a	Fidelity ^b
Template d	A (D-1)				
dTTP	4.2 ± 0.5	40 ± 10	$1.1 imes10^{-1}$		
dATP	0.0235 ± 0.0003	156 ± 7	$1.5 imes10^{-4}$	700	$1.4 imes10^{-3}$
dCTP	0.019 ± 0.001	70 ± 10	$2.7 imes10^{-4}$	390	$2.6 imes 10^{-3}$
dGTP	0.043 ± 0.003	170 ± 40	$2.5 imes 10^{-4}$	420	$2.4 imes 10^{-3}$

TABLE 5: Kinetic parameters of nucleotide incorporation into D-1 DNA catalyzed by full-length yPol η at 23°C.

^aCalculated as $(k_p/K_d)_{\text{correct}}/(k_p/K_d)_{\text{incorrect}}$.

^bCalculated as $(k_p/K_d)_{\text{incorrect}}/[(k_p/K_d)_{\text{correct}} + (k_p/K_d)_{\text{incorrect}}]$.

~4 s⁻¹ for both yPol η enzymes, but the misincorporation rate was 3- to 23-fold faster for truncated yPol η . This rate enhancement for truncated yPol η is partially offset by a greater discrimination at the apparent ground-state binding level so that the fidelity of truncated yPol η was only 3-folder lower than that of full-length yPol η .

3.6. Effect of the Nonenzymatic C-Terminus of yPoln on Its Polymerase Activity. Our above studies demonstrated that the C-terminus of yPol η enhances this enzyme's DNA binding affinity and base substitution fidelity by 2- and 3-fold, respectively. These results suggest that the nonenzymatic, C-terminal region of yPoln (Figure 1) has a mild impact on the N-terminal polymerase domain and its activity. This conclusion is inconsistent with previous studies which have qualitatively demonstrated that mutations or deletions in the UBZ domain or PIP motif do not affect polymerase activity [35–37]. However, these reported qualitative assays are not sufficiently sensitive to detect the small perturbation on polymerase activity as described in this paper. The presence of the C-terminal 119 residues of yPol η may either interact with DNA, slightly alter the conformation of the polymerase domain, or both (see above discussion), thereby enhancing its DNA binding affinity and polymerase fidelity.

3.7. Kinetic Comparison among Y-Family DNA Polymerases. The fidelity of several Y-family DNA polymerases synthesizing undamaged DNA has been determined by employing steady-state [38-48], pre-steady-state [28, 30, 49-53], or M13-based mutation assays [39, 41, 42, 45, 54, 55]. From these studies, the fidelity ranges from 10^0 to 10^{-4} . Under steady-state reaction conditions, the base substitution fidelity of yPol η and human Pol η has been measured to be in the range from 10^{-2} to 10^{-4} and 10^{-2} to 10^{-3} , respectively [38, 40], which is similar to our pre-steady-state kinetic results. Consistently, $Pol\eta$ displays the highest substrate specificity for the dCTP: dG base pair under both steady-state and presteady-state reaction conditions (Table 3 and unpublished data, Brown and Suo) [38, 40]. This may seem surprising, since Pol_{η} participates in the efficient bypass of UV-induced DNA damage such as a *cis-syn* thymine-thymine dimer (i.e., a dATP:dT base pair) [15-20, 56, 57]. However, Poln has also been shown to be efficient at bypassing guanine-specific damage such as 8-oxo-7,8-dihydro-dG [58, 59], 1,2-cisdiammineplatinum(II)-d(GpG) intrastrand cross-links [60-63], and various N2-dG lesions [64, 65].

Among the four eukaryotic Y-family DNA polymerases (i.e., Pol η , DNA polymerase κ , DNA polymerase ι (Pol ι), and Rev1), Rev1 exhibits low fidelity on undamaged DNA due to its strong preference for inserting dCTP [46, 52] while Poli has an unusual preference for dGTP:dT mispairs over dATP:dT due to Hoogsteen base pair formation [51, 69]. Interestingly, the lowest fidelity base pair for truncated $yPol\eta$ was dGTP:dT (Table 3). This observation likely results from the formation of a wobble base pair. The two hydrogen bonds established in the wobble base pair may enhance the catalytic efficiency of yPol η since hydrogen bonding is important for the efficiency and accuracy of $yPol\eta$ [70]. Also noteworthy, the truncated versions of eukaryotic Y-family DNA polymerases have been used for many biochemical studies in literature. Based on our quantitative kinetic analysis of yPol η , these results suggested the nonenzymatic regions of Y-family DNA polymerases do not alter the polymerase activity significantly.

3.8. Fidelity Comparison among Various DNA Polymerase Families. As a Y-family DNA polymerase, yPoln displays low fidelity on undamaged DNA (Tables 3 and 5) [38]. In contrast, replicative DNA polymerases in the A- and B-families have a polymerization fidelity that is 1–3 orders of magnitude greater than the Y-family DNA polymerases (Table 6). DNA polymerases with higher fidelity are more proficient at using the ground-state binding affinity to discriminate between a correct and incorrect dNTP. The Y-family DNA polymerases provide little to no discrimination based on the K_d difference while replicative DNA polymerases discriminate up to almost three orders of magnitude. This lack of selection in the ground state by the Y-family DNA polymerases may be due to the relatively loose and solvent-accessible active site which has minimal contacts with the nascent base pair [11, 21, 71]. Moreover, nucleotide selection by the Y-family DNA polymerases in the ground state may be mainly governed by Watson-Crick base pairing, since the calculated $\Delta\Delta G$ values (0.95–1.7 kcal/mol) are similar to the free energy differences between correct and incorrect base pairs (0.3-1.0 kcal/mol at 37°C) at the primer terminus based on DNA melting studies (Table 6) [72]. However, with $\Delta\Delta G$ values ≥ 3.0 kcal/mol, the replicative DNA polymerases harness the additional 2.0 kcal/mol of energy from other sources such as a tight active site or close contacts with the nascent base pair. One common fidelity checkpoint among DNA polymerases is the varying rate differences between a matched and mismatched

Polymerase	Polymerase Family	Fidelity ^a	K_d Difference ^b	k_p Difference ^b	$\Delta\Delta G (\text{kcal/mol})^{c}$
Truncated yPol η^{d}	Y	$3.7 imes 10^{-4}$ to $2.9 imes 10^{-2}$	3 to 68	5 to 220	1.6
Dpo4 ^e	Y	$1.5 imes 10^{-4}$ to $3.2 imes 10^{-3}$	1 to 18	240 to 1700	0.95
rPolβ ^f	Х	$1.1 imes 10^{-5}$ to $5.9 imes 10^{-4}$	35 to 342	28 to 708	3.0
PolB1 exo- ^g	В	$3.5 imes10^{-6}$ to $1.2 imes10^{-4}$	109 to 918	4 to 589	3.7
hPoly ^h	А	$4.6 imes 10^{-7}$ to $2.9 imes 10^{-4}$	42 to 900	39 to 12000	3.4

TABLE 6: Comparison of base substitution fidelity for various DNA polymerases.

^aCalculated as $(k_p/K_d)_{\text{incorrect}}/[(k_p/K_d)_{\text{correct}} + (k_p/K_d)_{\text{incorrect}}]$. ^bCalculated as defined in equation (8). ^cCalculated using equation (7). ^dAt 23°C (this work). ^eAt 37°C [50]. ^fAt 37°C [66]. ^gAt 37°C, excluding the fidelity contribution from the 3' \rightarrow 5' exonuclease activity [67]. ^hAt 37°C, excluding the fidelity contribution from the 3' \rightarrow 5' exonuclease activity [67].

base pair. These large differences may correspond to different rate-limiting steps (e.g., protein conformational change, or phosphodiester bond formation) during nucleotide incorporation [9, 30, 71]. For yPol η , kinetic data suggest that correct and incorrect dNTPs are limited by a conformational step preceding chemistry, although, additional studies are needed to confirm these results [28].

4. Conclusions

This work presents the mechanistic basis of the base substitution fidelity of yPol η on undamaged DNA, which examined all possible dNTP:dN base pair combinations for the first time. yPol η discriminates against incorrect nucleotides at both the ground-state nucleotide binding and incorporation steps. Furthermore, base stacking contributes to tighter binding for a misincorporation. Finally, the 119 residues at the C-terminus have a mild impact on the kinetic mechanism of yPol η .

Abbreviations

- BSA: Bovine serum albumin
- dNTP: 2'-deoxynucleoside 5'-triphosphate
- Dpo4: Sulfolobus solfataricus P2 DNA polymerase IV
- dPTP: Pyrene 5'-triphosphate
- EMSA: Electrophoretic mobility shift assay
- HPoly: Human mitochondrial DNA polymerase gamma
- PCNA: Proliferating cell nuclear antigen
- PIP: PCNA-interacting peptide
- PolB1: Exonuclease-deficient DNA polymerase B1 from sulfolobus solfataricus P2
- Pol*i*: DNA polymerase iota
- rPol β : Rat DNA polymerase beta
- TBE Tris/boric acid/EDTA
- UBZ: Ubiquitin-binding zinc finger
- YPoln: Saccharomyces cerevisiae DNA polymerase eta.

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