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Research Article

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

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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 (10⁶ 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′ → 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...
DNA template. Thus, it is important to understand the mechanism and fidelity of the Y-family DNA polymerases.

Saccharomyces cerevisiae DNA polymerase η (yPolη), a Y-family DNA polymerase, is critical for the error-free bypass of UV-induced DNA damage such as a cis-syn thymine-thymine dimer [15–19]. To date, Polη remains the only Y-family DNA polymerase with a confirmed biological function [20]. yPolη 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 yPolη’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 yPolη, it is unclear if the C-terminal residues 514–632 interact with DNA and contribute to the polymerase function of yPolη. Using pre-steady-state kinetic techniques, we have measured the base-substitution fidelity of full-length and truncated yPolη (Figure 1) catalyzing nucleotide incorporation into undamaged DNA. In addition, we have determined the DNA binding affinity of both full-length and truncated yPolη. Our results show that the C-terminus of yPolη 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: [γ-[32P]] ATP, MP Biomedicals (Solon, OH); BioSpin columns, Bio-Rad Laboratories (Hercules, 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 [γ-[32P]]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 yPolη (1–513) containing an N-terminal MGSSH₄SSGLVPRGSH tag was purified as described previously [24]. The full-length yPolη (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 yPolη (320 nM) and 5′-[32P]-labeled D-1 DNA (480 nM) was mixed with dTTP-Mg²⁺ (100 μM). To measure the dissociation rate of the yPolη-DNA binary complex, a preincubated solution of yPolη (50 nM) and 5′-[32P]-labeled D-1 DNA (100 nM) was mixed with a molar excess of unlabeled D-1 DNA (2.5 μM) for various time intervals prior to initiating the polymerization reaction with dTTP-Mg²⁺ (150 and 400 μM for truncated and full-length yPolη, resp.) for 15 s. For single-turnover kinetic assays, a preincubated solution of yPolη (150 nM) and 5′-[32P]-labeled DNA (30 nM) was mixed with an incoming dNTP-Mg²⁺ (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 (KₐDNA) of the yPolη-DNA binary complex was determined using two techniques. First, an electrophoretic mobility shift assay (EMSA) was employed by adding increasing concentrations of yPolη (10–450 nM) into a fixed concentration of 5′-[32P]-labeled D-1 DNA (10 nM) in

### Table 1: Sequences of DNA substrates.

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-1</td>
<td>5′-CGAGCCGTCCACCACTCA-3′</td>
</tr>
<tr>
<td>D-6</td>
<td>3′-GCCGTCACGTTGTTGAGATGCAGACTGTTACGCGAGG-5′</td>
</tr>
<tr>
<td>D-7</td>
<td>5′-CGCCGGCATTGTTGAGATGCAGACTGTTACGCGAGG-3′</td>
</tr>
<tr>
<td>D-8</td>
<td>3′-GCCGTCACGTTGTTGAGATGCAGACTGTTACGCGAG-5′</td>
</tr>
<tr>
<td>F-8</td>
<td>5′-CGCCGGCATTGTTGAGATGCAGACTGTTACGCGAGG-3′</td>
</tr>
<tr>
<td>BE1</td>
<td>5′-ATGAGTTCAGACTCAT-3′</td>
</tr>
<tr>
<td>BE2</td>
<td>3′-TCTCAACCGTGGAT-5′</td>
</tr>
<tr>
<td>BE3</td>
<td>5′-CTGAGTTCAGCTCAT-3′</td>
</tr>
<tr>
<td>BE4</td>
<td>3′-ACCTCAACCGTGGAT-5′</td>
</tr>
<tr>
<td></td>
<td><em>The template base highlighted in bold is unique to each strand and X denotes 2-aminopurine.</em></td>
</tr>
</tbody>
</table>
buffer A. The solution established equilibrium during a 20-
minute incubation period. Then, the binary complex was
separated from unbound DNA using a 4.5% native polyacry-
lamide 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 yPolη (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
Yvon Horiba). Emission background from the bu-

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):

\[ [\text{Product}] = A\left[1 - \exp(-k_{txt}t) + k_{sxt}t\right]. \]  

A represents the fraction of active enzyme, \( k_{txt} \) represents
the observed burst rate constant, and \( k_{sxt} \) represents the observed
steady-state rate constant.

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):

\[ [E \cdot DNA] = 0.5 \left( K_{d}^{DNA} + E_0 + D_0 \right) 
- 0.5 \left( \left( K_{d}^{DNA} + E_0 + D_0 \right)^2 - 4E_0D_0 \right)^{1/2}. \]  

\( D_0 \) is the DNA concentration.

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

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

\( F_{\text{max}} \) and \( F_{\text{min}} \) 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:

\[ [\text{Product}] = A\left[\exp(-k_{\text{off}}t)\right] + C. \]  

A represents the reaction amplitude, \( k_{\text{off}} \) 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 (\( k_{\text{obs}} \)):

\[ [\text{Product}] = A\left[1 - \exp(-k_{\text{obs}}t)\right]. \]  

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

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

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

\[ \Delta G = \frac{K_d^{\text{incorrect}}}{K_d^{\text{correct}}} \]  

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

3. Results and Discussion

Previously, transient state kinetic techniques have been
used to characterize full-length yPolη at 30°C [28]. There-
fore, we first performed a burst assay (see Section 2) to ensure
that our purified proteins, truncated and full-length yPolη
(Figure 1), behaved in a similar manner at 23°C. Compared
to wild-type yPolη, the truncated construct contains only
the polymerase domain (Figure 1). A preincubated solution
of yPolη (320 nM) and 5’-[32P]-labeled 21/41 mer D-1
DNA (480 nM) was mixed with dTTP-Mg2+ (100 μM) 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 full-
length yPolη at 30°C [28] and other DNA polymerases
[23, 29, 30].

3.2. The C-Terminal 119 Residues Slightly Enhance DNA Bind-
ing Affinity of yPolη. The equilibrium dissociation constant
for the binary complex of yPolη-DNA (\( K_{d}^{DNA} \)) was measured
to determine if the C-terminus of yPolη 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 yPolη (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 yPolη concentration which resolved a $K_d^{\text{DNA}}$ of 16 ± 1 nM (Figure 2(b) and Table 2). Under similar reaction conditions, the $K_d^{\text{DNA}}$ of truncated yPolη 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 yPolη (Table 2).

To corroborate these estimated $K_d^{\text{DNA}}$ values, we measured the true $K_d^{\text{DNA}}$ for the yPolη-DNA 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 flanked 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. 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 yPolη 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 yPolη concentration, and were fit to (4) which yielded DNA dissociation rates ($k_{\text{off}}$) of 0.008 ± 0.001 s$^{-1}$ and 0.0041 ± 0.0008 s$^{-1}$ for truncated and full-length yPolη, respectively (Table 2).

<table>
<thead>
<tr>
<th>Kinetic Parameter</th>
<th>Truncated yPolη</th>
<th>Full-length yPolη</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{on}}$ ($\text{M}^{-1} \text{s}^{-1}$)$^a$</td>
<td>0.62</td>
<td>0.59</td>
</tr>
<tr>
<td>$k_{\text{off}}$ (s$^{-1}$)</td>
<td>0.008 ± 0.001</td>
<td>0.0041 ± 0.0008</td>
</tr>
<tr>
<td>$K_d^{\text{DNA}}$ (nM)$^b$</td>
<td>34 ± 3</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>$K_d^{\text{DNA}}$ (nM)$^c$</td>
<td>13 ± 5</td>
<td>7 ± 4</td>
</tr>
</tbody>
</table>

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

$^b$Estimated using EMSA.

$^c$Measured using a fluorescence titration assay.
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 yPolη. Interestingly, we have previously demonstrated that the preferred nucleotide for template-independent nucleotide incorporation was in the range of $10^{-2}$ to $10^{-4}$ which translates into 1 to 10,000 nucleotide incorporations per 100 to 1,000 nucleotide incorporations (Table 3). Depending on the mispair, truncated yPolη 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 yPolη's fidelity, the equation for polymerase fidelity can be simplified as follows:

$$ Fidelity = \frac{\left(\frac{k_p}{K_d}\right)_{\text{incorrect}}}{\left(\frac{k_p}{K_d}\right)_{\text{correct}} + \left(\frac{k_p}{K_d}\right)_{\text{incorrect}}} = \frac{\left(\frac{k_p}{K_d}\right)_{\text{incorrect}}}{\left(\frac{k_p}{K_d}\right)_{\text{correct}}} = (\text{rate difference})^{-1} (\text{binding affinity difference})^{-1}. $$

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 yPolη'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.
Figure 3: Concentration dependence on the pre-steady-state rate constant of nucleotide incorporation catalyzed by truncated yPol\(\eta\). (a) A preincubated solution of truncated yPol\(\eta\) (150 nM) and 5\(^{-}\)\(^{32}\)P\]-labeled D-7 DNA (30 nM) was mixed with dATP-Mg\(^{2+}\) (0.4 \(\mu\)M, ○; 0.8 \(\mu\)M, □; 2 \(\mu\)M, ■; 4 \(\mu\)M, □; 8 \(\mu\)M, ▲; 16 \(\mu\)M, △; 40 \(\mu\)M, ●; 80 \(\mu\)M, ○) 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, \(k_{\text{obs}}\). (b) The \(k_{\text{obs}}\) values were plotted as a function of dATP concentration. The data (●) 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 \(\mu\)M.

Table 3: Kinetic parameters of nucleotide incorporation into D-DNA catalyzed by truncated yPol\(\eta\) at 23°C.

<table>
<thead>
<tr>
<th>dNTP</th>
<th>(k_p) (s(^{-1}))</th>
<th>(K_d) ((\mu)M)</th>
<th>(k_p/K_d) ((\mu)M(^{-1})s(^{-1}))</th>
<th>Discrimination Factor(^a)</th>
<th>Fidelity(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Template dA (D-1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dTTP</td>
<td>3.9 ± 0.2</td>
<td>15 ± 2</td>
<td>2.6 \times 10^{-1}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dATP</td>
<td>0.089 ± 0.005</td>
<td>80 ± 20</td>
<td>1.1 \times 10^{-3}</td>
<td>230</td>
<td>4.3 \times 10^{-3}</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.43 ± 0.06</td>
<td>210 ± 60</td>
<td>2.0 \times 10^{-3}</td>
<td>130</td>
<td>7.8 \times 10^{-3}</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.15 ± 0.01</td>
<td>80 ± 20</td>
<td>1.9 \times 10^{-3}</td>
<td>140</td>
<td>7.2 \times 10^{-3}</td>
</tr>
<tr>
<td><strong>Template dG (D-6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dCTP</td>
<td>15.6 ± 0.3</td>
<td>11.2 ± 0.8</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dATP</td>
<td>0.071 ± 0.002</td>
<td>138 ± 9</td>
<td>5.1 \times 10^{-4}</td>
<td>2700</td>
<td>3.7 \times 10^{-4}</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.116 ± 0.006</td>
<td>80 ± 10</td>
<td>1.5 \times 10^{-3}</td>
<td>960</td>
<td>1.0 \times 10^{-3}</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.92 ± 0.07</td>
<td>330 ± 40</td>
<td>2.8 \times 10^{-3}</td>
<td>500</td>
<td>2.0 \times 10^{-3}</td>
</tr>
<tr>
<td><strong>Template dT (D-7)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dATP</td>
<td>6.9 ± 0.4</td>
<td>17 ± 3</td>
<td>4.1 \times 10^{-1}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dCTP</td>
<td>1.00 ± 0.04</td>
<td>210 ± 20</td>
<td>4.8 \times 10^{-3}</td>
<td>85</td>
<td>1.2 \times 10^{-2}</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.55 ± 0.01</td>
<td>46 ± 3</td>
<td>1.2 \times 10^{-2}</td>
<td>30</td>
<td>2.9 \times 10^{-2}</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.62 ± 0.02</td>
<td>280 ± 20</td>
<td>2.2 \times 10^{-3}</td>
<td>180</td>
<td>5.4 \times 10^{-3}</td>
</tr>
<tr>
<td><strong>Template dC (D-8)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dGTP</td>
<td>6.3 ± 0.1</td>
<td>6.8 ± 0.4</td>
<td>9.3 \times 10^{-1}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dATP</td>
<td>0.087 ± 0.003</td>
<td>90 ± 10</td>
<td>9.7 \times 10^{-4}</td>
<td>960</td>
<td>1.0 \times 10^{-3}</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.127 ± 0.007</td>
<td>200 ± 30</td>
<td>6.4 \times 10^{-4}</td>
<td>1500</td>
<td>6.9 \times 10^{-4}</td>
</tr>
<tr>
<td>dTTP</td>
<td>1.39 ± 0.06</td>
<td>460 ± 40</td>
<td>3.0 \times 10^{-3}</td>
<td>310</td>
<td>3.3 \times 10^{-3}</td>
</tr>
</tbody>
</table>

\(^a\)Calculated as (\(k_p/K_d\))\(_{\text{correct}}\)/ (\(k_p/K_d\))\(_{\text{incorrect}}\).

\(^b\)Calculated as (\(k_p/K_d\))\(_{\text{incorrect}}\)/ ([\(k_p/K_d\)]\(_{\text{correct}}\) + (\(k_p/K_d\))\(_{\text{incorrect}}\)).
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 yPolη can catalyze template-independent nucleotide incorporation of dATP or dPTP (Figure 4) onto four palindromic, blunt-end 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 yPolη 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 $K_a$ values of dATP were 3- to 5-fold smaller with a purine than 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 yPolη-blunt-end DNA. This base-stacking effect is more dramatic for dPTP incorporation onto blunt-end DNA because the apparent $K_a$ 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 yPolη-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 yPolη. In addition, the base-stacking effect and van der Waals interactions may stabilize the ternary complex of yPolη-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 $K_a$, 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 yPolη. The base substitution fidelities of full-length and truncated yPolη may differ because the C-terminal, nonenzymatic regions may alter the polymerization fidelity. For example, the proline-rich 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 yPolη influences polymerization fidelity, we measured the pre-steady-state kinetic parameters for dNTP incorporation into D-1 DNA (template da) catalyzed by full-length yPolη (Table 5). The fidelity was calculated to be in the range of (1.4 to 2.6) $\times 10^{-3}$ for full-length yPolη (Table 5). Relative to the fidelity of truncated yPolη with D-1 (Table 3), full-length yPolη has a 3-fold higher fidelity. Therefore, the C-terminus of yPolη slightly affects the base substitution fidelity. Moreover, truncated yPolη discriminated between a correct and incorrect dNTP by ~30-fold on average based on the $k_p$ difference while the discrimination for full-length yPolη was ~170-fold on average for incorporation into D-1 DNA (Tables 3 and 5). The incorporation rate constant for correct dTTP was

<table>
<thead>
<tr>
<th>DNA (Terminal base pair)</th>
<th>dNTP</th>
<th>$k_p$ (s$^{-1}$)</th>
<th>$K_a$ (µM)</th>
<th>$k_p/K_a$ (µM$^{-1}$ s$^{-1}$)</th>
<th>Efficiency Ratio $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BE1 (dT : dA)</td>
<td>dATP</td>
<td>0.026 ± 0.002</td>
<td>1200 ± 200</td>
<td>2.2 × 10$^{-5}$</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dPTP</td>
<td>1.27 ± 0.08</td>
<td>60 ± 10</td>
<td>2.1 × 10$^{-2}$</td>
<td>980</td>
</tr>
<tr>
<td>BE2 (dA : dT)</td>
<td>dATP</td>
<td>0.036 ± 0.002</td>
<td>220 ± 30</td>
<td>1.6 × 10$^{-4}$</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dPTP</td>
<td>0.68 ± 0.03</td>
<td>23 ± 3</td>
<td>3.0 × 10$^{-2}$</td>
<td>180</td>
</tr>
<tr>
<td>BE3 (dG : dC)</td>
<td>dATP</td>
<td>0.0087 ± 0.0003</td>
<td>360 ± 30</td>
<td>2.4 × 10$^{-5}$</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dPTP</td>
<td>0.22 ± 0.01</td>
<td>9 ± 2</td>
<td>2.4 × 10$^{-2}$</td>
<td>1000</td>
</tr>
<tr>
<td>BE4 (dC : dG)</td>
<td>dATP</td>
<td>0.032 ± 0.001</td>
<td>930 ± 70</td>
<td>3.4 × 10$^{-5}$</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dPTP</td>
<td>0.74 ± 0.03</td>
<td>12 ± 2</td>
<td>6.2 × 10$^{-2}$</td>
<td>1800</td>
</tr>
</tbody>
</table>

$^a$Calculated as $(k_p/K_a)_{dPTP}/(k_p/K_a)_{dATP}$

Figure 4: Chemical structure of a nonnatural nucleotide analog, dPTP.
~4 s\(^{-1}\) for both \(\text{yPol}\eta\) enzymes, but the misincorporation rate was 3- to 23-fold faster for truncated \(\text{yPol}\eta\). This rate enhancement for truncated \(\text{yPol}\eta\) is partially offset by a greater discrimination at the apparent ground-state binding level so that the fidelity of truncated \(\text{yPol}\eta\) was only 3-folder lower than that of full-length \(\text{yPol}\eta\).

### 3.6. Effect of the Nonenzymatic C-Terminus of \(\text{yPol}\eta\) on Its Polymerase Activity

Our above studies demonstrated that the C-terminus of \(\text{yPol}\eta\) 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 \(\text{yPol}\eta\) (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 \(\text{yPol}\eta\) 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 \(\text{yPol}\eta\) and human \(\text{Pol}\eta\) 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, \(\text{Pol}\eta\) displays the highest substrate specificity for the dCTP:dG base pair under both steady-state and pre-steady-state reaction conditions (Table 3 and unpublished data, Brown and Suo) [38, 40]. This may seem surprising, since \(\text{Pol}\eta\) 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, \(\text{Pol}\eta\) has also been shown to be efficient at bypassing guanine-specific damage such as 8-oxo-7,8-dihydro-dG [58, 59], 1,2-cis-diammineplatinum(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., \(\text{Pol}\eta\), DNA polymerase \(\kappa\), DNA polymerase \(\iota\) (Pol\(\iota\)), and Rev1), Rev1 exhibits low fidelity on undamaged DNA due to its strong preference for inserting dCTP [46, 52] while Pol\(\iota\) has an unusual preference for dGTP:dT mismatches over dATP:dT due to Hoogsteen base pair formation [51, 69]. Interestingly, the lowest fidelity base pair for truncated \(\text{yPol}\eta\) 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 \(\text{yPol}\eta\) since hydrogen bonding is important for the efficiency and accuracy of \(\text{yPol}\eta\) [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 \(\text{yPol}\eta\), 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, \(\text{yPol}\eta\) 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
of yPol binding for a misincorporation. Finally, the 119 residues at steps. Furthermore, base stacking contributes to tighter both the ground-state nucleotide binding and incorporation rate-limiting steps (e.g., protein conformational change, or 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 on undamaged DNA, which examined all possible dNTP:dN base pair combinations for the first time. yPol 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.

### 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
- HPV: Human mitochondrial DNA polymerase gamma
- PCNA: Proliferating cell nuclear antigen
- PIP: PCNA-interacting peptide
- PolB: Exonuclease-deficient DNA polymerase B from Sulfolobus solfataricus P2
- Pol: DNA polymerase iota
- rPolβ: Rat DNA polymerase beta
- TBE: Tris/boric acid/EDTA
- UBZ: Ubiquitin-binding zinc finger
- YPol: Saccharomyces cerevisiae DNA polymerase eta.

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

<table>
<thead>
<tr>
<th>Polymerase</th>
<th>Polymerase Family</th>
<th>Fidelity</th>
<th>$k_\text{f}$ Difference</th>
<th>$k_\text{p}$ Difference</th>
<th>$\Delta\text{G}$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Truncated yPol$^a$</td>
<td>Y</td>
<td>$3.7 \times 10^{-4}$ to $2.9 \times 10^{-2}$</td>
<td>3 to 68</td>
<td>5 to 220</td>
<td>1.6</td>
</tr>
<tr>
<td>Dpo4$^a$</td>
<td>Y</td>
<td>$1.5 \times 10^{-4}$ to $3.2 \times 10^{-4}$</td>
<td>1 to 18</td>
<td>240 to 1700</td>
<td>0.95</td>
</tr>
<tr>
<td>rPolβ$^b$</td>
<td>X</td>
<td>$1.1 \times 10^{-5}$ to $5.9 \times 10^{-4}$</td>
<td>35 to 342</td>
<td>28 to 708</td>
<td>3.0</td>
</tr>
<tr>
<td>PolB1 exo-$^c$</td>
<td>B</td>
<td>$3.5 \times 10^{-6}$ to $1.2 \times 10^{-4}$</td>
<td>109 to 918</td>
<td>4 to 589</td>
<td>3.7</td>
</tr>
<tr>
<td>hPol$^b$</td>
<td>A</td>
<td>$4.6 \times 10^{-7}$ to $2.9 \times 10^{-4}$</td>
<td>42 to 900</td>
<td>39 to 12000</td>
<td>3.4</td>
</tr>
</tbody>
</table>

$^a$Calculated as $(k_p/K_d)_{\text{correct}}/[(k_p/K_d)_{\text{correct}}+(k_p/K_d)_{\text{incorrect}}]$. $^b$Calculated as defined in equation (8). $^c$Calculated using equation (7). $^d$At 23°C (this work).

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, 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].

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