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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 (yPolη), 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 yPolη which contains only the polymerase domain. In the absence of yPolη’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 yPolη may interact with DNA and slightly alter the conformation of the polymerase domain during catalysis. In general, yPolη 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.
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 yPol (1–513) containing an N-terminal DNA template. Thus, it is important to understand the mechanism and fidelity of the Y-family DNA polymerases.

Saccharomyces cerevisiae DNA polymerase η (yPol), 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, Pol η remains the only Y-family DNA polymerase with a confirmed biological function [20]. yPol 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 yPol'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, it is unclear if the C-terminal residues 514–632 interact with the yPol DNA binary complex, a preincubated solution of yPol`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, it is unclear if the C-terminal residues 514–632 interact with the DNA and contribute to the polymerase function of yPol. Using pre-steady-state kinetic techniques, we have measured the base-substitution fidelity of full-length and truncated yPol (Figure 1) catalyzing nucleotide incorporation into undamaged DNA. In addition, we have determined the DNA binding affinity of both full-length and truncated yPol. Our results show that the C-terminus of yPol has a minor effect on the DNA binding affinity and the base substitution fidelity of this lesion bypass DNA polymerase.

**Table 1:** Sequences of DNA substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-1</td>
<td>5′-CCGACGGCCTCCACCAACTCA-3′</td>
</tr>
<tr>
<td>D-2</td>
<td>3′-GGTGGGAGTTGGATCGACCTAGTTACGGCAGG-5′</td>
</tr>
<tr>
<td>D-3</td>
<td>5′-CCGACGGCCTCCACCAACTCA-3′</td>
</tr>
<tr>
<td>D-4</td>
<td>3′-GGTGGGAGTTGGATCGACCTAGTTACGGCAGG-5′</td>
</tr>
<tr>
<td>D-5</td>
<td>5′-CCGACGGCCTCCACCAACTCA-3′</td>
</tr>
<tr>
<td>D-6</td>
<td>3′-GGTGGGAGTTGGATCGACCTAGTTACGGCAGG-5′</td>
</tr>
<tr>
<td>D-7</td>
<td>5′-CCGACGGCCTCCACCAACTCA-3′</td>
</tr>
<tr>
<td>D-8</td>
<td>3′-GGTGGGAGTTGGATCGACCTAGTTACGGCAGG-5′</td>
</tr>
<tr>
<td>D-9</td>
<td>5′-CCGACGGCCTCCACCAACTCA-3′</td>
</tr>
<tr>
<td>D-10</td>
<td>3′-GGTGGGAGTTGGATCGACCTAGTTACGGCAGG-5′</td>
</tr>
<tr>
<td>BE1</td>
<td>5′-ATGGTTGCAACCTCAT-3′</td>
</tr>
<tr>
<td>BE2</td>
<td>3′-TACCTAACGTGGATGT-5′</td>
</tr>
<tr>
<td>BE3</td>
<td>5′-CTGGATTGCAACCTCAT-3′</td>
</tr>
<tr>
<td>BE4</td>
<td>3′-AATCTAACGTGGATGT-5′</td>
</tr>
<tr>
<td>BE5</td>
<td>5′-CCGACGGCCTCCACCAACTCA-3′</td>
</tr>
<tr>
<td>BE6</td>
<td>3′-GGTGGGAGTTGGATCGACCTAGTTACGGCAGG-5′</td>
</tr>
<tr>
<td>BE7</td>
<td>5′-ATGGTTGCAACCTCAT-3′</td>
</tr>
<tr>
<td>BE8</td>
<td>3′-TACCTAACGTGGATGT-5′</td>
</tr>
<tr>
<td>BE9</td>
<td>5′-CTGGATTGCAACCTCAT-3′</td>
</tr>
<tr>
<td>BE10</td>
<td>3′-AATCTAACGTGGATGT-5′</td>
</tr>
</tbody>
</table>

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

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 (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 yPol DNA binary complex, a preincubated solution of yPol (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 yPol, resp.) for 15 s. For single-turnover kinetic assays, a preincubated solution of yPol (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 (Kd,DNA) of the yPol-DNA binary complex was determined using two techniques. First, an electrophoretic mobility shift assay (EMSA) was employed by adding increasing concentrations of yPol (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 20-minute 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� (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. Data Analysis. For the pre-steady-state burst assay, the product concentration was graphed as a function of time. 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 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):

\[
[\text{Product}] = A[1 - \exp(-k_1t) + k_2t].
\] (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):

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

\(D_0\) 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:

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

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

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

\[
\Delta\Delta G = RT \ln \left[\frac{(K_d)^{incorrect}}{(K_d)^{correct}}\right]. \] (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 yPolη Display Biphasic Kinetics. Previously, transient state kinetic techniques have been used to characterize full-length yPolη at 30°C [28]. Therefore, 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 \(S^\prime-1^{[32P]}\)-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 Binding 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 yPol (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 concentration which resolved a $K_d$ of 16 ± 1 nM (Figure 2(b) and Table 2). Under similar reaction conditions, the $K_d$ of truncated yPol was estimated to be 34 ± 3 nM, a binding affinity (1/$K_d$) value that is 2-fold weaker than that of full-length yPol (Table 2).

To corroborate these estimated $K_d$ values, we measured the true $K_d$ for the yPol-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 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. The fluorescence titration assay allows yPol to associate and dissociate during data collection. In contrast, EMSA does not maintain a constant equilibrium because dissociated yPol 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 which indicates that the C-terminal 119 amino acid residues of yPol slightly enhance the binding of the enzyme to DNA.

Next, we directly measured the rate of DNA dissociation from the yPol-DNA complex (see Section 2). A preincubated solution of yPol (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. 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 and full-length yPol, respectively (Table 2 and Scheme 1). Interestingly, the rate of DNA dissociation from full-length yPol is 2-fold slower than that from truncated yPol, which indicated that the C-terminus of yPol may slightly contribute to this polymerase’s DNA binding affinity.

Based on the measured $K_d$ from Figure 2(c) and $k_{off}$ values, the apparent second-order association rate constant (k$\text{on} = k_{off}/K_d$) of the binary complex yPol-DNA was calculated to be 0.62 and 0.59 μM$^{-1}$s$^{-1}$ for truncated and full-length yPol, respectively (Table 2). These similar $k_{on}$ values indicate that the slightly stronger DNA binding affinity of full-length yPol 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 yPol slightly hinder the dissociation of DNA from the binary complex yPol-DNA. This hindrance is through either direct physical interactions between the C-terminus of yPol and DNA, modulation of the conformation of the polymerase domain by the C-terminus of yPol, or both.

### Table 2: Rate and equilibrium dissociation constants for the binary complex yPol-DNA at 23°C.

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

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

$^b$Estimated using EMSA.

$^c$Measured using a fluorescence titration assay.

### 3.3. Base Substitution Fidelity of Truncated yPol

Since a pre-steady-state burst was observed for truncated yPol, 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 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 (150 nM) and 5′-[32P]-labeled D-7 DNA (30 nM) was mixed with varying concentrations of dATP-Mg$^{2+}$ (0.4–80 µM) and quenched with EDTA at
various times (see Section 2). A plot of product concentration versus time was fit to (5) to extract the observed rate constant ($k_{\text{obs}}$) for dATP incorporation (Figure 3(a)). Then, the $k_{\text{obs}}$ values were plotted as a function of dATP concentration and fit to a hyperbolic equation (6) which resolved a $k_p$ of $6.9 \pm 0.4$ 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:DNA base pair combinations were determined under single-turnover conditions and were used to calculate the substrate specificity constant ($k_p/K_d$), discrimination factor ($(k_p/K_d)_{\text{correct}}/((k_p/K_d)_{\text{incorrect}})$), and fidelity ($((k_p/K_d)_{\text{correct}}/((k_p/K_d)_{\text{correct}} + (k_p/K_d)_{\text{incorrect}}))$) of truncated yPol$\eta$ (Table 3).

Overall, the base substitution fidelity of truncated yPol$\eta$ 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$\eta$ 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$\eta$’s fidelity, the equation for polymerase fidelity can be simplified as follows:

$$\text{Fidelity} = \frac{(k_p/K_d)_{\text{incorrect}}}{((k_p/K_d)_{\text{correct}} + (k_p/K_d)_{\text{incorrect}})}$$

$$\approx \frac{(k_p/K_d)_{\text{incorrect}}}{(k_p/K_d)_{\text{correct}}}$$

$$= [\frac{(k_p)_{\text{incorrect}}}{(k_p)_{\text{correct}}}]^{-1} \left[\frac{(K_d)_{\text{correct}}}{(K_d)_{\text{incorrect}}}\right]^{-1}$$

$$= (\text{rate difference})^{-1} (\text{binding affinity difference})^{-1}.$$  

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 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$\eta$. Interestingly, we have previously demonstrated that the preferred nucleotide for template-independent nucleotide

![Figure 2: Equilibrium dissociation constant for full-length yPol$\eta$.](image)
Figure 3: Concentration dependence on the pre-steady-state rate constant of nucleotide incorporation catalyzed by truncated yPolη. (a) A preincubated solution of truncated yPolη (150 nM) and 5'-[32P]-labeled D-7 DNA (30 nM) was mixed with dATP-Mg2+ (0.4 μM, ○; 0.8 μM, ●; 2 μM, ■; 4 μM, □; 8 μM, ▲; 16 μM, △; 40 μM, ◆; 80 μM, ◊) 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 (●) were then fit to a hyperbolic equation, yielding a $k_p$ of $6.9 \pm 0.4$ s$^{-1}$ and a $K_d$ of $17 \pm 3$ μM.

Table 3: Kinetic parameters of nucleotide incorporation into D-DNA catalyzed by truncated yPolη at 23°C.

<table>
<thead>
<tr>
<th>dNTP</th>
<th>$k_p$ (s$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$k_p/K_d$(μM$^{-1}$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 × 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 × 10$^{-3}$</td>
<td>230</td>
<td>4.3 × 10$^{-3}$</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.43 ± 0.06</td>
<td>210 ± 60</td>
<td>2.0 × 10$^{-3}$</td>
<td>130</td>
<td>7.8 × 10$^{-3}$</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.15 ± 0.01</td>
<td>80 ± 20</td>
<td>1.9 × 10$^{-3}$</td>
<td>140</td>
<td>7.2 × 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 × 10$^{-4}$</td>
<td>2700</td>
<td>3.7 × 10$^{-4}$</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.116 ± 0.006</td>
<td>80 ± 10</td>
<td>1.5 × 10$^{-3}$</td>
<td>960</td>
<td>1.0 × 10$^{-3}$</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.92 ± 0.07</td>
<td>330 ± 40</td>
<td>2.8 × 10$^{-3}$</td>
<td>500</td>
<td>2.0 × 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 × 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 × 10$^{-3}$</td>
<td>85</td>
<td>1.2 × 10$^{-2}$</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.55 ± 0.01</td>
<td>46 ± 3</td>
<td>1.2 × 10$^{-2}$</td>
<td>30</td>
<td>2.9 × 10$^{-2}$</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.62 ± 0.02</td>
<td>280 ± 20</td>
<td>2.2 × 10$^{-3}$</td>
<td>180</td>
<td>5.4 × 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 × 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 × 10$^{-4}$</td>
<td>960</td>
<td>1.0 × 10$^{-3}$</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.127 ± 0.007</td>
<td>200 ± 30</td>
<td>6.4 × 10$^{-4}$</td>
<td>1500</td>
<td>6.9 × 10$^{-4}$</td>
</tr>
<tr>
<td>dTTP</td>
<td>1.39 ± 0.06</td>
<td>460 ± 40</td>
<td>3.0 × 10$^{-3}$</td>
<td>310</td>
<td>3.3 × 10$^{-3}$</td>
</tr>
</tbody>
</table>

$^a$Calculated as $(k_p/K_d)_{correct}/(k_p/K_d)_{incorrect}$

$^b$Calculated as $(k_p/K_d)_{incorrect}/((k_p/K_d)_{correct}+(k_p/K_d)_{incorrect})$.
Table 4: Kinetic parameters for nucleotide incorporation onto blunt-end DNA catalyzed by truncated yeast Polη at 23°C.

<table>
<thead>
<tr>
<th>DNA (Terminal base pair)</th>
<th>dNTP</th>
<th>$k_p$ (s$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$k_p/K_d$ (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 $(k_p/K_d)_{dPTP}/(k_p/K_d)_{dATP}$

3.5. Base Substitution Fidelity of Full-Length yPol. The base substitution fidelities of full-length and truncated yPol 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 yPol 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 (Table 5). The fidelity was calculated to be in the range of (1.4 to 2.6) × 10$^{-5}$ for full-length yPol (Table 5). Relative to the fidelity of truncated yPol with D-1 (Table 3), full-length yPol has a 3-fold higher fidelity. Therefore, the C-terminus of yPol slightly affects the base substitution fidelity. Moreover, truncated yPol 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 was ∼170-fold on average for incorporation into D-1 DNA (Tables 3 and 5). The incorporation rate constant for correct dTPP was catalyzed by Dpo4 [26]. Although neither dATP nor dPTP forms any hydrogen bonds with a template base when bound by yPol-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 yPol. In addition, the base-stacking effect and van der Waals interactions may stabilize the ternary complex of yPol-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).
qualitatively demonstrated that mutations or deletions in the N-terminal polymerase domain and its activity. This is consistent with previous studies which have demonstrated that mutations or deletions in the C-terminal polymerase domain and its activity. This is consistent with previous studies which have demonstrated that mutations or deletions in the C-terminal polymerase domain and its activity.

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 $10^{-6}$ to $10^{-4}$. Under steady-state reaction conditions, the base substitution fidelity of yPolη increases as the template dA (D-1) and its activity. This is consistent with previous studies which have demonstrated that mutations or deletions in the C-terminal polymerase domain and its activity.

<table>
<thead>
<tr>
<th>dNTP</th>
<th>$k_p$ (s$^{-1}$)</th>
<th>$K_d$ (µM)</th>
<th>$k_p/K_d$ (µM$^{-1}$ s$^{-1}$)</th>
<th>Discrimination Factor$^a$</th>
<th>Fidelity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTTP</td>
<td>4.2 ± 0.5</td>
<td>40 ± 10</td>
<td>1.1 ± 10$^{-1}$</td>
<td>700</td>
<td>1.4 × 10$^{-3}$</td>
</tr>
<tr>
<td>dATP</td>
<td>0.0235 ± 0.0003</td>
<td>156 ± 7</td>
<td>1.5 ± 10$^{-4}$</td>
<td>390</td>
<td>2.6 × 10$^{-3}$</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.019 ± 0.001</td>
<td>70 ± 10</td>
<td>2.7 ± 10$^{-4}$</td>
<td>420</td>
<td>2.4 × 10$^{-3}$</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.043 ± 0.003</td>
<td>170 ± 40</td>
<td>2.5 ± 10$^{-4}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

$^b$Calculated as $\left( k_p/K_d\right)_{\text{incorrect}}/\left( k_p/K_d\right)_{\text{correct}} + \left( k_p/K_d\right)_{\text{incorrect}}$.

Among the four eukaryotic Y-family DNA polymerases (i.e., Polη, DNA polymerase ι, DNA polymerase ε (Polε), and Rev1), Rev1 exhibits low fidelity on undamaged DNA due to its strong preference for inserting dCTP [46, 52] while Polη 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 yPolη 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 yPolη since hydrogen bonding is important for the efficiency and accuracy of yPolη [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 yPolη, these results suggested the nonenzymatic regions of Y-family DNA polymerases do not alter the polymerase activity significantly.

3.6. Effect of the Nonenzymatic C-Terminus of yPolη on Its Polymerase Activity. Our above studies demonstrated that the C-terminus of yPolη 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 yPolη (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 yPolη 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 $10^{-9}$ to $10^{-4}$. Under steady-state reaction conditions, the base substitution fidelity of yPolη has been measured to be in the range of $10^{-2}$ to $10^{-3}$, respectively [38, 40], which is similar to our pre-steady-state kinetic results. Consistently, Polη displays the highest substrate specificity for the dCTP : dG base pair under both steady-state and pre-steady-state reaction conditions (Table 6 and unpublished data, Brown and Suo) [38, 40]. This may seem surprising, since Polη 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, Polη 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].

3.8. Fidelity Comparison among Various DNA Polymerase Families. As a Y-family DNA polymerase, yPolη 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 ΔΔG 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 ΔΔG 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...
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 yPolγ, 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 yPolγ on undamaged DNA, which examined all possible dNTP:dN base pair combinations for the first time. yPolγ 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 yPolγ.

Abbreviations

BSA: Bovine serum albumin
dNTP: 2′-deoxyribose 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
Polβ: Exonuclease-deficient DNA polymerase B from Sulfolobus solfataricus
Polγ: DNA polymerase iota
rPolβ: Rat DNA polymerase beta
TBE: Tris/boric acid/EDTA
UBZ: Ubiquitin-binding zinc finger
YPolγ: Saccharomyces cerevisiae DNA polymerase eta.

Acknowledgments

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References


