Fluorescence Resonance Energy Transfer Studies of DNA Polymerase β

THE CRITICAL ROLE OF FINGERS DOMAIN MOVEMENTS AND A NOVEL NON-COVALENT STEP DURING NUCLEOTIDE SELECTION*

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Background: DNA Pol β participates in base excision repair by choosing correct dNTP to fill single-nucleotide gaps in DNA.

Results: Pol β experiences a non-covalent step with correct dNTP selection.

Conclusion: Correct and incorrect dNTP incorporation by Pol β are different.

Significance: FRET-based system of Pol β elucidates a mechanism of substrate choice necessary for understanding the molecular basis of human disease.

During DNA repair, DNA polymerase β (Pol β) is a highly dynamic enzyme that is able to select the correct nucleotide opposite a templating base from a pool of four different deoxynucleoside triphosphates (dNTPs). To gain insight into nucleotide selection, we use a fluorescence resonance energy transfer (FRET)-based system to monitor movement of the Pol β fingers domain during catalysis in the presence of either correct or incorrect dNTPs. By labeling the fingers domain with (2-iodoacetyl)aminomethyl)naphthalene-1-sulfonic acid (IAEDANS) and the DNA substrate with Dabcyl, we are able to observe rapid fingers closing in the presence of correct dNTPs as the IAEDANS comes into contact with a Dabcyl-labeled, one-base gapped DNA. Our findings show that not only do the fingers close after binding to the correct dNTP, but that there is a second conformational change associated with a non-covalent step not previously reported for Pol β. Further analyses suggest that this conformational change corresponds to the binding of the catalytic metal into the polymerase active site. FRET studies with incorrect dNTP result in no changes in fluorescence, indicating that the fingers do not close in the presence of incorrect dNTP. Together, our results show that nucleotide selection initially occurs in an open fingers conformation and that the catalytic pathways of correct and incorrect dNTPs differ from each other. Overall, this study provides new insight into the mechanism of substrate choice by a polymerase that plays a critical role in maintaining genome stability.

Approximately 20,000 DNA lesions are removed per human cell per day via the base excision repair (BER) pathway (1). Short patch BER functions in the removal of single-base damages from DNA. BER is initiated by removal of a damaged base by a DNA glycosylase (2). A monofunctional glycosylase generates an abasic (AP) site that is processed by apurinic/apyrimidinic AP endonuclease 1 (APE1), which nicks the sugar-phosphate backbone to generate a 3’ OH and a 5’ deoxyribose phosphate, which is removed by Pol β. Bifunctional glycosylases catalyze excision of the damaged base and AP site removal, leaving a 3’ deoxyribose phosphate and a 5’ phosphate. APE1 catalyzes removal of the 3’ deoxyribose phosphate to generate a 3’ OH. In both cases, Pol β fills in the gap and XRCC1-Ligase IIIα or Ligase I seals the remaining nick in the DNA (3).

Pol β plays an important role in ensuring the fidelity of DNA synthesis during repair. The 39-kDa protein is a member of the X-family of DNA polymerases and has four domains: the 8-kDa lyase, thumb, palm, and fingers domains. Whereas the other three domains are responsible for DNA binding and catalytic activity, the fingers domain (residues 262–335) forms the nucleotide binding pocket and thus plays a central role in nucleotide selection (4–7). A series of crystal structures of Pol β suggest that binding of dNTPs induces a conformational change whereby helix N of the fingers domain moves closer to the DNA (8). This conformational change occurs rapidly to form the active site, which catalyzes phosphodiester bond formation (also known as the chemistry step). Product release is considered to be the rate-limiting step (Scheme 1) (9).

Pol β is known to misincorporate an incorrect nucleotide once in every 10,000 base insertions (10). The efficiency of incorrect incorporation is increased in several Pol β variants that exhibit a mutator phenotype (11–15). Previous fluorescence studies with Pol β performed by us (16) and others (17–19) observed movements of 3′-phosphate backbone to generate a 3′ OH and a 5′ deoxyribose phosphate, which is removed by Pol β. Bifunctional glycosylases catalyze excision of the damaged base and AP site removal, leaving a 3′ deoxyribose phosphate and a 5′ phosphate. APE1 catalyzes removal of the 3′ deoxyribose phosphate to generate a 3′ OH. In both cases, Pol β fills in the gap and XRCC1-Ligase IIIα or Ligase I seals the remaining nick in the DNA (3).

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associated with fingers closing. Many of these studies were conducted on a recessed DNA substrate, which is not the physiologically relevant substrate for Pol β (20, 21), as Pol β prefers single nucleotide-gapped DNA. Given these limitations, we developed a FRET-based approach to monitor the movements of the fingers domain during DNA synthesis by Pol β, using a single nucleotide-gapped DNA substrate.

Our results reveal that there is a rapid closing of the fingers domain in the presence of the correct nucleotide, followed by an additional and not previously identified non-covalent step that precedes chemistry and product release. The conformational changes we observe with correct dNTP incorporation are absent in the presence of the incorrect dNTP, suggesting that Pol β catalysis is different for incorporation of correct versus incorrect nucleotides.

**EXPERIMENTAL PROCEDURES**

Deoxyoligonucleotides and DNA sequences used in this paper are described (Table 1).

*Generation of C239S/C267S/V303C Pol β—*Wild-type (WT) human DNA polymerase β contains three endogenous cysteine residues. The mutations of Cys-239 and Cys-267 to serines were introduced into a tagless, human WT Pol β (modified pET28a) by site-directed mutagenesis (Stratagene) and did not affect Pol β function (Table 2). An additional residue change by site-directed mutagenesis converted Val-303 to Cys to facilitate thiol-reactive labeling with (((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS).

**TABLE 1**

One base pair gap DNA substrate used in this study

| DNA sequence | Substrate |
|--------------|-----------|
| 5′-GCCCTGCCAGCCGCTGAGTCATGCAACATGTCGATTCCCGGGAGG-5′ | T:3′OH* |
| 5′-GCCCTGCCAGCCGCTGAGTCATGCAACATGTCGATTCCCGGGAGG-5′ | T:3′OH* |
| 5′-GCCCTGCCAGCCGCTGAGTCATGCAACATGTCGATTCCCGGGAGG-5′ | T:3′H+ |
| 5′-GCCCTGCCAGCCGCTGAGTCATGCAACATGTCGATTCCCGGGAGG-5′ | T:3′H+ |
| 5′-GCCCTGCCAGCCGCTGAGTCATGCAACATGTCGATTCCCGGGAGG-5′ | T:3′H+ |

* T:3′OH is the extendable DNA substrate without a Dabcyl residue.
* T:3′OH is extendable DNA substrate with a Dabcyl residue at the −8 position from the templating base.
* T:3′H is non-extendable DNA substrate without a Dabcyl residue.
* T:3′H is non-extendable DNA substrate with a Dabcyl residue at the −8 position from the templating base.
nm-32P-labeled, one-base gapped DNA (Table 1) were mixed with 100 μM dCTP and 10 mM MgCl₂. All mixtures contained reaction buffer E (50 mM Tris-HCl, pH 8, 100 mM NaCl, and 10% glycerol) and were allowed to react for 0.2 to 3 s at 37 °C. Reactions were quenched with 0.5 M EDTA and 90% formamide sequencing dye. Radioactive products were separated on a 20% denaturing polyacrylamide gel, observed on a Storm 860 PhosphorImager, and quantified based on a 20% denaturing polyacrylamide gel, observed on a Storm 860 PhosphorImager, and quantified based on n and n+1 products using ImageQuant software. The data were fitted to the following biphasic burst equation using Prism 6 GraphPad software.

\[
\text{[Product]} = A(1 - e^{-k_{obs}})
\]

(Eq. 2)

Here, \(k_{obs}\) is the observed rate at each concentration of enzyme.

**TABLE 2**

Activity of modified Pol β

| Pol β       | Residue 178 | Residue 239 | Residue 267 | \(k_{ss}\) \(\text{a} \) |
|------------|-------------|-------------|-------------|----------------|
| WT         | C           | C           | C           | 13 ± 2         |
| 178S/239S  | Ser         | Ser         | C           | Highly unstable |
| 178S/267S  | Ser         | Ser         | C           | No burst        |
| 239S/267S  | C           | Ser         | C           | 12 ± 2         |
| 178S/239S/267S | Ser       | Ser         | C           | No burst        |

* Reactions were carried out with 100 μM Pol β and 300 μM T-3′OH using 100 μM dCTP as described under “Experimental Procedures.”

**FIGURE 1.** Crystal structure of human DNA polymerase β [Protein Data Bank codes 3ISB and 4KLE]. A. Pol β contains four domains (3ISB): the 8-kDa domain (yellow), thumb domain (blue), palm domain (green), and the fingers domain (red). B. A rotation of 180° of the crystal structure reveals the distance between the IAEDANS and Dabcyl to be 43.1 Å in the open, binary complex (3ISB). Residue 303 on the fingers domain is labeled with IAEDANS and the DNA is labeled with Dabcyl-T 8 bases away from the gap (–8). C. In the closed, ternary complex, IAEDANS and Dabcyl-T have a calculated distance (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC) of 33.7 Å (4KLE).

**Pol β FRET Studies of Fingers Movement during Nucleotide Selection**

Single-turnover Experiments—Pol β-AEDANS and radiolabeled T(-6)D:3′OH were assayed on the KinTek apparatus to determine \(k_{pol}\) and \(K_{d（dNTP）}\) at 37 °C. Correct nucleotide (dCTP) was titrated from 0.5 to 200 μM over a range of 0.03 to 10 s with 500 nm Pol β and 50 nm DNA. Radioactive products were separated on a denaturing polyacrylamide gel and quantified as described above. Using Prism 6, the data were fitted to a single exponential equation, where \(k_{obs}\) is the observed rate constant at each concentration of dNTP. The rates (\(k_{obs}\)) are plotted against concentration of dNTP and fitted to the hyperbolic equation,

\[
k_{obs} = \frac{k_{pol}[dNTP]}{K_{d（dNTP）} + [dNTP]}
\]

(Eq. 3)

\(k_{pol}\) is the maximum rate of polymerization and \(K_{d（dNTP）}\) is the equilibrium dissociation constant for the incoming dNTP.

Circular Dichroism—The secondary structural characteristics of unlabeled Pol β, Pol β-AEDANS, and Cys-178 were analyzed by circular dichroism as previously described (24). The ellipticity of 3 μM labeled protein solution was normalized to 3 μM unlabeled Pol β.

Fluorescence Emission Scan—The fluorescence of Pol β-AEDANS (600 nm) was observed at room temperature on the Photon Technology International spectrofluorometer in Buffer F (50 mM Tris-HCl, pH 7, 10 mM MgCl₂, and 1 mM EDTA). The sample was excited at 336 nm and an emission scan was performed from 380 to 650 nm. The DNA substrate T(-8)D:3′H was added to the protein mixture and scanned, followed by the addition of 100 μM dCTP (correct) or 500 μM dATP (incorrect).

Stopped-flow Fluorescence—In all experiments, the sample was excited at 336 nm, the emissions were filtered with a 400-nm filter, and the voltage was fixed at 400 V. Pol β-AEDANS was assayed along with Dabcyl-labeled DNA substrate (see figure legends for specific DNA used) in the stopped-flow SX-19 (Applied Photophysics) at 37 °C. Under single turnover conditions, 500 nm Pol β was co-incubated with 200 nm T(-8)D:3′H or 100 nm T(-8)D:3′OH in buffer E and then rapidly mixed in the stopped-flow instrument with various concentrations of dNTP as detailed in the Figure legends. In some experiments, MgCl₂ was exchanged for CaCl₂. Data collection began as soon as mixing occurred (pre-trigger setting). Fluorescent signal artifacts from the flow and mixing of the solutions were removed from the traces (0–0.0308 s) based on the results...
from standardized reactions (25). A logarithmic data collection of 500 time points over 10 s was evaluated for each mixing event with five traces per dNTP concentration that were averaged together.

**KinTek Explorer Fitting**—Rate constants were determined by KinTek Explorer Professional Version 3 (26, 27). Raw data were imported into the program as a concentration series. The data were plotted a logarithmic time scale and plot residuals were used to ensure a precise fit. In addition, concentration series offset (addition/subtraction) was added to the simulation. The observables were noted as,

\[ a \times (ED + b \times EDN + c \times END...) + \text{offset}_1a \]  

(Eq. 4)

where the observables reflect the presence of protein [E]. The \( K_{d(dNTP)} \) was confirmed using the following equation,

\[ K_{d(dNTP)} = \frac{k_-}{k_+} \]  

(Eq. 5)

where \( k_- \) and \( k_+ \) correspond to the rate constants determined by KinTek Explorer.

A nonlinear regression equation was used to initially estimate the fit of the raw stopped-flow data. In instances where the non-covalent step was absent (Fig. 8A), the analytic function \( f(t) = a_1 \times \exp(-b_1 \times t) + c \), was used. When the non-covalent step was present (Figs. 5, A and B, and 8B), the function \( f(t) = a_1 \times \exp(-b_1 \times t) + a_2 \times \exp(-b_2 \times t) + c \) was used. Data for extendable DNA (Fig. 7A) was estimated to a quadruple exponential equation. The data were fit by KinTek Explorer to the best possible fit and constrained by standard error and chemical quench data (Fig. 3) (26). The non-covalent step was further defined by one-dimensional FitSpace calculation of 5.5–25.5 s\(^{-1}\) (27).

**RESULTS**

**IAEDANS-labeled Pol β and Dabcyl-labeled DNA**—We designed a FRET-based approach for monitoring fingers movement of Pol β, based on previous work with DNA polymerase I (23). We chose to label Pol β with the thiol reactive IAEDANS in the fingers domain, and to label the DNA template with Dabcyl. Quenching of fluorescence will occur upon finger closure, as the fingers move toward the Dabcyl in the DNA, according to crystal structures of the open binary and closed ternary complexes. Pol β contains three endogenous Cys residues at positions 178, 239, and 267; none of which would be appropriate for accurately reporting the fingers movement. An initial characterization of Pol βC239S and Pol βC267S, in which either Cys-239 or Cys-267 were altered to Ser, or of the double mutant (C239S,C267S) indicated that altering these residues to Ser would not affect the overall Pol β function (Table 2). However, alteration of Cys-178 to Ser affected activity (Table 2), and we thus elected not to alter this residue. Val-303, located on a small loop in the fingers domain, was mutated to a Cys residue that could react with the IAEDANS (Fig. 1A). The placement of the Dabcyl quencher on the DNA was carefully selected to maintain an appropriate Förster distance of about 40 Å. A Dabcyl-T was inserted eight bases downstream (−8) from the single nucleotide gap, which provided an optimal distance for observing FRET (Table 1 and Fig. 1, A and B).
The Labeled V303C Pol β Variant Has Kinetics Properties Similar to WT Pol β—The variant of Pol β bearing three mutations (C239S, C267S, and V303C) termed V303C, was subcloned into a tagless pET28a expression vector and expressed in Escherichia coli. The purified protein was labeled with IAEDANS similar to previously published protocols (23, 28). A control protein termed Cys-178, which harbors only the C239S and C267S alterations with the endogenous Cys-178 maintained, was expressed, purified, and labeled in the same manner. Analysis of a 10% SDS-PAGE gel containing samples of both the IAEDANS-treated V303C and Cys-178 protein preparations detected either by UV or Coomassie staining suggests that the thiol reaction occurs at the Cys-303 position and that Cys-178 is not labeled with IAEDANS (Fig. 2, A and B) presumably because it is buried. To further confirm protein structure, circular dichroism spectroscopy suggests labeled protein preparations retained similar secondary structure characteristics of unlabeled WT Pol β (Fig. 2C).

Pol β V303C-AEDANS was assessed for pre-steady-state burst activity using the extendable T(−8)D:3′OH substrate. The burst rate observed for Pol β V303C-AEDANS (14 s−1) is similar to the burst rate of unlabeled Pol β (13 s−1) on nonlabeled DNA (Fig. 3A and Table 2). This rapid rate of 14 s−1 suggests that alteration of Cys-239 and Cys-267 to Ser, as well as the labeling with IAEDANS at position 303, did not affect the overall rate of DNA synthesis catalyzed by Pol β and that the overall rate-limiting step was still product release. We also performed experiments under single turnover conditions using a ratio of 10:1 protein:DNA. These conditions were determined empirically by incubating various concentrations of protein with 100 nM DNA and 100 μM dNTP for various times (11). As shown in Table 3 and Fig. 3, B and C, the kobs, kpol, and KD(dNTP) for Pol β V303C-AEDANS are similar to unlabeled Pol β (29). In combination, our results show that the Pol β V303C-AEDANS has kinetic properties similar to WT Pol β that are unaffected by the IAEDANS label.

FRET Is Observed in Steady-state with Pol β V303C-AEDANS and Dabcyl-labeled DNA—To determine whether we could observe a FRET signal with the Pol β V303C-AEDANS and Dabcyl-labeled DNA, we characterized steady-state fluorescence. Upon excitation of the Pol β V303C-AEDANS alone at 336 nm, an emission scan reveals a peak at ~490 nm (Fig. 4A, red). Upon addition of the non-extendable T(−8)D:3′H Dabcyl-labeled DNA substrate we observed a quench in fluorescence intensity, indicating the formation of a binary complex (Fig. 4A, blue). Upon addition of the correct dNTP (dCTP) we observed greater fluorescence quenching, suggesting that Pol β is in a ternary complex in which the fingers domain moves closer to the Dabcyl in the DNA (Fig. 4A, green). Finally, when an incorrect dNTP (dATP) is added to binary Pol β we observe less of a fluorescence quench compared with that of correct dNTP (Fig. 4A, black). The same experiment was repeated with non-Dabcyl-labeled DNA T:3′H (Fig. 4B). There was little decrease in the fluorescence intensity of Pol β-AEDANS upon addition of T:3′H and both correct or incorrect dNTP, indicating that the quenching seen in Fig. 4A is due to the IAEDANS/Dabcyl FRET pair coming within the Förster radii. These results demonstrate that we are able to observe a FRET signal between the polymerase and DNA in the presence of dNTPs.

Stopped-flow Fluorescence Reveals a Novel Non-covalent Step in the Pol β Catalytic Pathway—To characterize movements of the fingers domain in the absence of phosphoryl transfer we performed stopped-flow fluorescence experiments in which the correct dNTP was mixed with Pol β V303C-AEDANS prebound to the non-extendable T(−8)D:3′H DNA substrate (Fig. 5A). In the presence of the correct nucleotide (dCTP), there is a rapid decrease in fluorescence suggesting that during the first 0.1 s, the fingers domain comes within close proximity of the Dabcyl-labeled DNA, such that a quench is observed. Similar traces were observed with extendable T(−8)D:3′OH DNA in
the presence of a dCpCpp, a non-hydrolyzable analog of dCTP (Fig. 5B).

The data obtained with the T(−8)D:3′H DNA substrate and correct dNTP were initially fitted with Prism 6 to both single- and double-exponential equations. The residuals indicated that a double-exponential equation was the better fit (Fig. 6, A and B), suggesting that upon binding correct dNTP, these two phases indicate that there are at least two movements. We then employed KinTek Explorer to globally fit all of the stopped-flow phases. The residuals suggested that a reduction in intensity was observed with incorporation of 500 μM incorrect dATP (black). B, steady-state fluorescence of Pol β-AEDANS (red) was repeated as described above with non-Dabcyl-labeled substrate T:3′H (blue) and correct (green) or incorrect (black) dNTP. The minor quenching that is observed is likely due to the presence of dNTP.

FIGURE 4. Steady-state fluorescence of Pol β-AEDANS in the presence of Dabcyl-DNA and dNTP. A, the fluorescence intensity of 600 μM Pol β V303C-AEDANS (red) is reduced when bound to 100 μM Dabcyl-DNA substrate T(−8)D:3′H (binary complex, blue). The addition of 100 μM correct dNTP (dCTP) opposite template G results in a further reduction in fluorescence intensity (green) suggesting a reduction in distance between the IAEDANS/Dabcyl pair. Less of a reduction in intensity was observed with incorporation of 500 μM incorrect dATP (black). B, steady-state fluorescence of Pol β-AEDANS (red) was repeated as described above with non-Dabcyl-labeled substrate T:3′H (blue) and correct (green) or incorrect (black) dNTP. The minor quenching that is observed is likely due to the presence of dNTP.

FIGURE 5. Stopped-flow fluorescence of Pol β V303C-AEDANS in the absence of chemistry demonstrates fingers closing. A, a solution of Pol β V303C-AEDANS and non-extendable Dabcyl-T DNA (T(−8)D:3′H) in a 2.5:1 protein to DNA ratio was rapidly mixed with increasing correct dCTP to give the final concentrations at the right. B, similar to the experiment in A, the dNTP substrate was replaced with the non-hydrolyzable dCpCpp analog and T(−8)D:3′OH was used for stopped-flow fluorescence experiments. In all experiments, n = 5. KinTek Explorer fits are represented by the dashed line.

Nucleotide Incorporation follows a Different Pathway for Correct Versus Incorrect Nucleotide—To examine the role of the fingers domain in nucleotide selection, stopped-flow experiments were performed under single turnover conditions with T(−8)D:3′OH and either correct (dCTP) or incorrect (dATP or dTTP) substrates. For correct incorporation, we observed initial quenching of fluorescence followed by a gradual recovery of fluorescence (Fig. 7A). Chemistry is occurring due to the presence of the 3′-OH and dCTP and the increase in fluorescence could reflect chemistry or steps subsequent to chemistry. Stopped-flow traces for incorrect dNTP incorporation significantly differ from those with correct dNTP. There was no change in fluorescence with an incorrect purine or pyrimidine, even when titrating up to 900 μM incorrect dNTPs (Fig. 7, B and C).

KinTek Explorer simulations suggest that the kinetics of correct incorporation are best described by a 5-step kinetic model for Pol β-AEDANS with extendable T(−8)D:3′OH DNA (Fig. 7A) in which Pol β proceeds through: step 2, dNTP binding (ED + N ⇄ EDN); step 3, a conformational change after recognition of the correct dNTP (EDN ⇄ END); step 3.1, a second conformational change (END ⇄ NDE); step 3.2, chemistry (NDE ⇄ EP); step 4, DNA dissociation and product release (EP ⇄ E + P). Due to the linear nature of the stopped-flow trace obtained with an incorrect dNTP (Fig. 7, B and C), we were unable to fit the data to an exponential equation or to model the data with KinTek Explorer.

Table 4 summarizes the estimated relative rates of the reactions in the presence of non-extendable and extendable DNA substrates. Parameters used to constrain our model include chemical quench data as well as 2-AP and stopped-flow rates derived using intrinsic tryptophan residues for Pol β (16, 30, 31). The rate constant k1 represents the dNTP binding event. Importantly, calculation of the Kd(dNTP) as described under “Experimental Procedures” (k−1/Kd(dNTP)) yielded a Kd(dNTP) of 1.9 to 3 μM, which is within the range of previously published Kd(dNTP) for Pol β (29, 32). We suggest that the k1 rate constant represents the fingers movement toward the DNA. We
suggest a value of change associated with the fingers closing and the 10 and 0.03 s (deviation of the dCTP. A subsequent step of EDN binds to a three-step kinetic scheme using KinTek Explorer.

The kinetic scheme below the graph defines EDN as initial, which represents a second non-covalent step following closing of the fingers, mainly because it occurs with both the extendable and non-extendable DNA substrates. The k_{+3} rate constant is most likely phosphodiester bond formation, because it corresponds to the rate of chemistry, k_{pol}, that we measure in rapid chemical quench flow assays. Furthermore, this rate constant is not present when the primer terminus is not extendable (Table 3). The k_{+4} rate constant is the overall rate-limiting step of the kinetic pathway and is similar to the steady-state rate previously measured by us and others (36, 37). This likely represents dissociation of Pol β from the DNA.

Scheme 2 summarizes these proposed kinetic steps for Pol β.

Characterization of the Non-covalent Step with Catalytically Inactive Ca^{2+}—To further explore the non-covalent step (k_{+3}), CaCl_2 was used in place of MgCl_2 in the stopped-flow experiments. Calcium has a larger ionic radius than Mg^{2+} and does not support phosphoryl transfer (38, 39). In the presence of non-extendable DNA and CaCl_2, we observed a fluorescence decrease upon binding to the correct dCTP (Fig. 8A) that could be described by a two-step model using KinTek Explorer (steps 2 and 3 of Scheme 2). In contrast, the fluorescence trace observed with extendable DNA (Fig. 8B) was best described by a three-step model (steps 2, 3, and 3.1 of Scheme 2). These results indicate that in the presence of extendable DNA, the initial fingers domain movement and the non-covalent step are intact in the presence of CaCl_2, but that the chemistry step and subsequent product release did not occur due to the inability of CaCl_2 to support phosphodiester bond formation. However, the non-covalent step (step 3.1 of Scheme 2) was not observed with CaCl_2 and non-extendable DNA. This suggests that the non-covalent step 3.1 requires the presence of a 3’-OH.

**DISCUSSION**

We have developed a FRET-based system for Pol β, which allows monitoring of movements of the fingers domain during DNA synthesis. Our results provide valuable insight into the movements of the fingers domain during catalysis. We observe that Pol β experiences at least two conformational changes that precede phosphodiester bond formation.

**Incorporation of Correct Versus Incorrect Nucleotide by Pol β Is Different**—In agreement with published work for Pol β as well as the A-family polymerases (unlike Y-family polymerases (9, 19, 23, 40, 41, 43–45), we observe that the initial global movement of the fingers domain is not rate-limiting and this initial domain movement likely plays a critical role in facilitating polymerization. Single molecule experiments have also suggested this to be the case for Sulfolobus solfataricus DNA polymerase B1 (46). Importantly, incorrect dNTP binding does not induce any conformational changes with either a pyrimidine or purine within the first 10 to 20 s of the reaction, using FRET to monitor the fingers domain movements. Our results, therefore, suggest that dNTP discrimination occurs at ground state dNTP binding at a point before the fingers have closed. Future fluorescence experiments with mutator variants of Pol β that map to a number of different regions of the protein (see for example, Refs. 47–50) will likely provide mechanistic insights into how recognition occurs at this point.
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FIGURE 7. The fingers domain closes in the presence of correct dNTP, but not in the presence of incorrect dNTP. A, Pol β V303C-AEDANS and extendable Dabcyl-T DNA (T(−8)D:3 OH) were mixed with increasing concentrations of correct dCTP in single turnover conditions. The stopped-flow fluorescence traces were globally fit to a five-step model. Misincorporation of Pol β V303C-AEDANS was analyzed using stopped-flow fluorescence, similar to A with the exception of increasing incorrect concentrations of dATP (B) and dTTP (C). In all experiments, n = 5. KinTek Explorer fits are represented by the dashed line.

TABLE 4
Kinetic rate constants for the Pol β V303C-AEDANS catalytic cycle during nucleotide incorporation

Based on Scheme 2 analyzed by KinTek Explorer.

| Rate constant* | Model* | T(−8)D:3′ H + Mg2++ | T(−8)D:3′ OH + dCpCpp | T(−8)D:3′ OH + Mg2++ | T(−8)D:3′ H + Ca2++ | T(−8)D:3′ OH + Ca2++ |
|----------------|--------|----------------------|------------------------|----------------------|----------------------|----------------------|
| k-1 | ED + N → EDN | 11.8 ± 0.64 | 5.79 ± 0.903 | 12.1 ± 0.885 | 35.6 ± 3.07 | 48.2 ± 3.58 |
| k-2 | EDN → ED | 97.8 ± 18.8 | 166 ± 22.2 | 121 ± 27.0 | 116 ± 11.4 | 224 ± 26.9 |
| k-3 | END → EDN | (0.01–3.5)‡ | ~4.02 × 10⁻¹⁰ | (0.00730–2.84) | (0.999–4.2) | ~7.94 × 10⁻⁷ |
| k-4 | NDE → END | 8.1 ± 5.5 | 7.89 ± 0.342 | 17.0 ± 0.179 | 11.9 ± 0.797 | ~4.50 × 10⁻⁷ |
| k-5 | NDE → EP | (0.06–6.2)‡ | 1.1 × 10⁻⁷ | 63 ± 11.1 | 8.92 | 0 |
| k-6 | EP → NDE | 1.28 ± 0.165 | 21.6 ± 4.15 | | | |
| k-7 | E + P → EP | 0.885 35.6 | 7.94 224 | 7.94 224 | 7.94 224 | 7.94 224 |

* Rate constants reported in s⁻¹ except where indicated by † (μM⁻¹ s⁻¹).
* Model used in KinTek Explorer program where E represents enzyme; D represents DNA; N represents nucleotide.
* The deviations from the rate constant shown are S.E. (26).
* Values represent the range.

SCHEME 2. Biochemical pathway model for KinTek Explorer simulations.

The binary complex of Pol β (E/DNAED) is in equilibrium (k₋₁/k₋₀) with the nucleotide (N) to form the ternary complex (EDN) and is represented by step 2 in the overall biochemical pathway suggested in Scheme 3. After formation of the ternary complex (EDN), the fingers domain moves closer to the DNA (END), signified by the rate constant k₋₂ (step 3). A second conformational change occurs in which there is a rearrangement of the active site (NDE), indicated by k₋₃ (a novel step 3.1). This rearrangement facilitates chemistry (EP) k₋₄ (step 3.2), followed by product release (step 4) k₋₅.

into the how fidelity is governed before closure of the fingers. Our previous work suggests that the hinge region plays a role in substrate discrimination (51–53) suggesting that the hinge may in some way “sense” the presence of correct versus incorrect dNTP during its initial contact with the fingers domain, and rotate toward a closed complex only if correct dNTP is bound.

A Novel Non-covalent Step in the Pol β Kinetic Pathway—The determination of rates from stopped-flow experiments was supported by chemical quench analysis. Our data, with all DNA substrates and metals, report a K₋₄(dNTP) of 1.9–3 μM dCTP. We measured the K₋₄(dNTP) for the labeled protein under single turnover conditions and found it to be 1.4 μM (Table 3), which is similar to k₋₄/k₋₀ determined with KinTek Explorer and is confirmed by our own previously published chemical quench data of human Pol β on this particular substrate (T3′OH-, Table 2, and Scheme 2, step 2) (29, 32). Step 3 corresponds to the rapid fingers closing, which is >100 s⁻¹ in the presence of Mg2++. We have constrained this step using previously published fast rate constants (16, 30, 31). Due to the complexity of the stopped-flow traces, we used KinTek Explorer to reveal an additional step that immediately precedes chemistry (step 3.1) that occurs with an upper limit of 25 s⁻¹ that has not been previously characterized. This step is remarkably absent with the non-extendable T(−8)D′3′ H DNA substrate in the presence of Ca²⁺ (Fig. 8A, Table 4). The new results emerging from this work call for an expansion of Scheme 1 for the pathway of nucleotide incorporation by Pol β, including a non-covalent step that may facilitate chemistry as shown in Scheme 3.

A non-covalent step has not been identified in previous studies with Pol β, although there is precedence for a non-covalent step in the kinetic pathways of the Klenow fragment and Klentaq1 (23, 54, 55) as well as the Y-family DNA polymerase, Dpo4 (40). Step 3.1 (step 3.1, Scheme 2) is of particular interest as it suggests a further rearrangement after fingers closing, but just prior to chemistry, which was not previously observed for Pol β using 2-AP and Trp.

We further explored the non-covalent step 3.1 by replacing Mg²⁺ with Ca²⁺. Calcium is larger than Mg²⁺ and generally cannot support phosphoryl transfer (38, 56). Pol β uses a two-metal ion mechanism (57, 58) for catalysis in which two metal ions participate in the reaction and coordinate the catalytic triad of three aspartate residues, Asp-190, Asp-192, and Asp-256. The catalytic metal A (MgA) lowers the pH of the incoming dNTP substrate. Metal B coordinates the non-bridging oxygens...
The non-covalent step is dependent on the presence of a free primer 3′OH group and the catalytic Mg\(^{2+}\). A, Pol β V303C-AEDANS and non-extendable Dabcyl-T DNA (T(−8)D3′H) were mixed with increasing concentrations of dNTP (correct, dCTP) and CaCl\(_2\) was substituted for MgCl\(_2\). B, Pol β V303C-AEDANS was analyzed using stopped-flow fluorescence, similar to A with the exception of T−8D3′OH, extendable DNA. In all experiments, \(n = 5\). KinTek Explorer fits are represented by the dashed line.

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