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Single-Molecule Investigation of Response to Oxidative DNA Damage by a Y-Family DNA Polymerase

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Abstract

Y-family DNA polymerases are known to bypass DNA lesions in vitro and in vivo and rescue stalled DNA replication machinery. Dpo4, a well-characterized model Y-family DNA polymerase, is known to catalyze translesion synthesis across a variety of DNA lesions including 8-oxo-7,8-dihydro-2'-deoxyguanine (8-oxo-dG). Our previous X-ray crystallographic, stopped-flow Förster resonance energy transfer (FRET), and computational simulation studies have revealed that Dpo4 samples a variety of global conformations as it recognizes and binds DNA. Here we employed single-molecule FRET (smFRET) techniques to investigate the kinetics and conformational dynamics of Dpo4 when it encountered 8-oxo-dG, a major oxidative lesion with high mutagenic potential. Our smFRET data indicated that Dpo4 bound the DNA substrate in multiple conformations, as suggested by three observed FRET states. An incoming correct or incorrect nucleotide affected the distribution and stability of these states with the correct nucleotide completely shifting the equilibrium towards a catalytically competent complex. Furthermore, the presence of the 8-oxo-dG lesion in the DNA stabilized both the binary and ternary complexes of Dpo4. Thus, our smFRET analysis provided a basis for the enhanced efficiency which Dpo4 is known to exhibit when replicating across from 8-oxo-dG.

Graphical Abstract

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SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Control experiments to verify FRET system and labeled enzyme activity. Dwell time, and transition density plots for damaged and undamaged DNA substrates. Selected smFRET trajectories. Nucleotide selectivity FRET distribution histograms.

ABBREVIATIONS

Sulfolobus solfataricus Dpo4; Y-family DNA polymerase; oxidative DNA lesion; 8-oxo-7, 8-dihydro-2'-deoxyguanine; single-molecule FRET; translesion synthesis (TLS)

One of the most common sources of endogenous DNA damage is aerobic respiration. This essential life process generates oxygen radicals, which are known to cause damage to DNA. For example, 8-oxo-7,8-dihydro-2'-deoxyguanine (8-oxo-dG), a major oxidative DNA lesion, is formed by the oxidation of the C8 atom of guanine. While structural studies have shown that 8-oxo-dG does not significantly distort the local structure of DNA,⁽¹⁻³⁾ the lesion remains particularly mutagenic by often being better accommodated in a polymerase active site following rotation about the *N*-glycosidic bond to adopt the unusual *syn*-conformation. While the *anti*-conformation of 8-oxo-dG forms a correct Watson-Crick base pair with correct dCTP, the *syn*-conformation readily forms Hoogsteen interactions with incorrect dATP.⁽⁴⁻⁶⁾ Unique interactions with 8-oxo-dG within the polymerase active site can either promote or limit misincorporation events.^(4, 7-11) If left unrepaired, 8-oxo-dG:dA mispairs will result in G→T transversion mutations, which are implicated in cancer induction.^(12, 13)

DNA lesions can also interfere with faithful DNA replication by stalling high-fidelity replicative DNA polymerases. While 8-oxo-dG does not completely block DNA synthesis, it does cause polymerase pausing in some instances.⁽¹⁴⁻²²⁾ It is proposed that this pausing initiates translesion DNA synthesis (TLS) via a polymerase switching mechanism that allows the cell to recruit Y-family DNA polymerases which are known for bypassing lesions *in vitro* and *in vivo*. Notably, the Y-family polymerases have been identified in all three domains of life, *e.g.* four in humans (DNA polymerases η , κ , ι , and Rev1), two in *Escherichia coli* (DNA polymerase IV and V), and one in *Sulfolobus solfataricus* (DNA polymerase IV (Dpo4)).

Pre-steady-state kinetic and crystallographic data indicate that Dpo4 preferentially incorporates dCTP opposite 8-oxo-dG with high efficiency due to stabilization of the *anti*-conformation of the lesion in its active site.^(10, 11, 23, 24) Recently, our real-time stopped-flow FRET studies have revealed how the individual domains of Dpo4 move during substrate binding and catalysis.⁽²⁵⁻²⁷⁾ Dpo4, akin to all canonical polymerases, contains three core polymerase domains denoted as Finger, Palm, and Thumb. Moreover, characteristic of all Y-family members, Dpo4 also contains an auxiliary domain termed the Little Finger (LF) which is connected to the Thumb domain by a highly flexible 14-amino acid residue peptide linker. The LF domain is known to impart unique DNA binding and lesion bypass capabilities to the Y-family polymerases.⁽²⁸⁾ While crystallographic investigation suggests that upon nucleotide binding, no large-scale domain movements exist from the binary to ternary structures,⁽²⁹⁾ our real-time stopped-flow FRET investigations illustrate global conformational changes in Dpo4 during nucleotide binding and incorporation onto undamaged DNA.⁽²⁵⁻²⁷⁾ These investigations permitted the expansion of the minimal kinetic mechanism for polymerase-catalyzed nucleotide incorporation.

Recently, single-molecule FRET (smFRET) methodologies have been employed to investigate the conformational dynamics of DNA polymerases and have illustrated how

domain motions impact substrate recognition and selectivity.^(30–36) Specifically, the role of the Finger domain in nucleotide selectivity has been well characterized.^(30, 33, 35) While the Finger domain of Dpo4 has not been exclusively implicated in nucleotide selection, it is part of synchronized, global domain motions which likely play an important role in nucleotide recognition and selectivity. Understanding the mechanism of nucleotide recognition and selectivity is of even greater importance since the Y-family DNA polymerases lack a proof-reading exonuclease domain. Here, our smFRET study revealed that Dpo4 existed in equilibrium among three FRET states that likely represent distinct structural conformations of Dpo4 and its positions on the undamaged or 8-oxo-dG-containing DNA substrates. The addition of an incoming nucleotide affected the distribution of the FRET states and the DNA binding stability of the polymerase. Our study further established a mechanism by which Dpo4 can faithfully and efficiently bypass a major oxidative lesion.

MATERIAL AND METHODS

Preparation of Protein and DNA

We selected a catalytically active mutant of Dpo4 used in previous work with mutations in the Finger domain (C31S, N70C) for the present study.^(25–27) The engineered Cys mutation allowed for site-specific fluorophore labeling (Figure 1B) which was carried out by incubation of Dpo4 with a 15-fold molar excess of Cy5-maleimide (GE Healthcare), overnight at 4°C in a buffer containing 50 mM Tris (pH 7.2), 150 mM NaCl, 0.5 mM TCEP, and 10% glycerol. Unincorporated free dye was then removed with Micro Bio-Spin columns (Bio-Rad). By measuring the absorbance at 280 nm and 650 nm, the ratio of protein concentration to dye concentration revealed a labelling efficiency of 91% for the reaction.

The 21-mer primer containing a 5'-biotin for surface immobilization as well as a 5-C6-amino-2'-deoxythymidine modification at the 9th base from the 3' terminus for Cy3-NHS-ester labelling, and control undamaged template were purchased from Integrated DNA Technologies. This labelling was performed according to the manufacturer's protocol (GE Healthcare) (Figure 1A). The oligonucleotide containing the 8-oxo-dG base was purchased from Midland Certified Reagent Company, Incorporated. The primer and template oligonucleotides were annealed to each other by heating to 80°C for 5 minutes followed by slow cooling to room temperature.

Steady-state Fluorescence Spectroscopy Assays

Fluorescence spectra were recorded on a Fluoromax-4 (HORIBA Jobin Yvon) at 20°C. Increasing amounts of Cy5-labeled Dpo4 (0–180 nM) were titrated into a 25 nM solution of the Cy3-labeled DNA substrate (undamaged or damaged) in a buffer containing 25 mM HEPES (pH 7.5), 5 mM CaCl₂, 25 mM NaCl and 10 % glycerol. The change in donor quantum yield was plotted against acceptor concentration. The resulting curve was fit to Equation 1 to yield the equilibrium dissociation constant for the interaction.

$$\Delta\phi = (\Delta\phi_T / 2D_0) \times \left\{ (K_D^{DNA} + E_0 + D_0) - 0.5 \left[(K_D^{DNA} + E_0 + D_0)^2 - 4E_0D_0 \right]^{1/2} \right\} \quad (\text{Eq. 1})$$

where ϕ is the change in the donor quantum yield, ϕ_T is the maximum change in donor quantum yield, D_0 is the total DNA concentration, E_0 is the total Dpo4 concentration, and K_D^{DNA} is the equilibrium dissociation constant.

Single-molecule Measurements

Single-molecule fluorescence experiments were conducted on a custom built prism-type total internal reflection microscope as described previously.⁽³⁵⁾ Imaging chambers were assembled from quartz slides and coverslips which were cleaned, passivated, biotinylated and coated with Neutravidin (0.2 mg/mL) following a standard, published protocol.⁽³⁷⁾ The Cy3-labeled DNA was flowed into the imaging chamber at a concentration sufficient to observe single, distinct immobilized molecules (20–40 pM). In addition to the Dpo4 binding buffer (50 mM HEPES (pH 7.6), 50 mM NaCl, 0.1 mM EDTA, 5 mM CaCl₂, and 0.1 mg/mL BSA), experiments were performed in an imaging solution containing an oxygen scavenging system (0.8% (w/v) d-glucose, 1 mg/mL glucose oxidase, and 0.04 mg/mL catalase) and 2 mM Trolox. Ca(II) was used as the divalent metal ion in place of Mg(II) to prevent the incorporation of dNTPs. Ca(II) in the polymerase active site has been shown to closely mimic Mg(II), but prevents or dramatically reduces dNTP incorporation.^(38, 39) Movies were recorded at 100 ms exposure time resolution, at 20°C, over several minutes upon introduction of 10–20 nM Cy5-labeled Dpo4 to the imaging chamber. The movies were then processed using IDL (ITT Visual Information Solutions) and analyzed by the use of custom data acquisition and analysis software which included a MATLAB (MathWorks) script necessary for correcting anti-correlated donor and acceptor time trajectories for background (Center for the Physics of Living Cells, University of Illinois at Urbana-Champaign).

Verification of the FRET System

To confidently correlate changes in FRET with molecular distance changes rather than unrelated photophysical artifacts, we performed several previously described control experiments.⁽⁴⁰⁾ We recorded movies of surface immobilized Cy3-DNA following excitation at 532 nm in the presence and absence of WT Dpo4 to show that the donor intensity remains constant until photobleaching between the experiments (Figure S1). Further, we recorded acceptor fluorescence upon direct excitation of 10 nM Cy5-Dpo4 with 638 nm laser light. A histogram composed of acceptor intensities from >100 time trajectories revealed one major distribution and, more notably, the absence of higher intensity distributions eliminating the possibility of multiple Dpo4 molecules binding per DNA substrate (Figure S2).

Single Molecule Data Analysis

From the donor and acceptor time trajectories, apparent FRET (E_{app}) was

$$\frac{I_A}{I_D + I_A} = E_{app} \quad (\text{Eq. 2})$$

calculated by Equation 2, where I_A is the acceptor intensity and I_D is the donor intensity. Notably, only fluorescence time trajectories with a single photobleaching event were selected for further processing in order to avoid ambiguity in removing background signal. FRET efficiency values from over 200 fluorescence time trajectories with acceptor intensities clearly above background level (as determined by the average background after donor photobleaching) were collected and binned from 0–1 to generate population histograms using a previously described thresholding approach.^(35, 41) The population distributions were fit to a sum of Gaussian functions using MATLAB, and the percent density of molecules in each respective population was calculated as the total area under each peak. Each histogram was fit with the minimum number of peaks necessary to give a visually acceptable fit. The fit was generally deemed acceptable if the percent error, calculated as the total difference between the experimental bin heights and the computed value of the fit at each bin center, divided by the sum of the bin heights, was less than 10%. However, given the limited signal-to-noise ratio of our single-molecule method and the overlap between FRET distributions, we did not want to bias our analysis to presume a certain number of FRET states. Therefore, to impartially verify our visual assignment of FRET states from the raw trajectory data and the accuracy of the Gaussian fits, we performed a probability based Hidden Markov modelling (HMM) analysis using HaMMMy software.⁽⁴²⁾ To further avoid biasing the results, the HaMMMy software was set to converge on the true number of FRET states in the data from initially guessing the maximum number of FRET states that the program allows (10). We analyzed a subset of single-molecule trajectories (100) for each smFRET experiment by HMM as the HaMMMy software was computationally limited in the number of traces that could be processed at one time under our experimental settings (i.e. 10 state model). The HaMMMy output files were then additionally processed using accompanying TDP software to generate transition density plots. Kinetic information describing the transitions between states was calculated through the TDP software. Briefly, the transition distributions were fit to Gaussian functions and the calculated peak centers and standard deviations were converted to kinetic rates and rate errors, respectively, by multiplying by the exposure time used during data acquisition (100 ms). HaMMMy and TDP were used primarily to complement and verify the results we obtained from the manual analysis of the complete data sets as well as to describe the kinetic rates connecting interconverting FRET states which would be difficult to acquire otherwise.

Dwell time analysis was performed as described previously.⁽³⁵⁾ Survivor functions were subsequently fit to single (Equation 3) or double (Equation 4) exponential decay equations.

$$f(t) = A \exp(-kt) \quad (\text{Eq. 3})$$

$$f(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) \quad (\text{Eq. 4})$$

where $f(t)$ is the fraction of molecule remaining bound after time t , A_1 and A_2 are the amplitudes of the phases, and k , k_1 , and k_2 are the rate constants of Dpo4 unbinding from

the DNA substrate. Fits to the data were evaluated based on the coefficient of determination (R^2) as well as visual inspection. To avoid over interpreting the results, all data were initially assumed to demonstrate single exponential decay behavior. If the R^2 value was less than 0.99 and the fitting curve failed to adequately represent the majority of data, a higher order exponential fit was considered necessary (Equation 4).

Kinetic Assays

Polymerase activity was assessed through a burst kinetic assay at 37°C in a reaction buffer containing 50 mM HEPES, (pH 7.5 at 37°C), 50 mM NaCl, 0.1 mg/mL BSA, 0.1 mM EDTA, 5 mM DTT, 10% glycerol, and 5 mM $MgCl_2$. Briefly, 60 nM of 5'- ^{32}P -labeled D-1 DNA substrate was pre-incubated with 15 nM of either WT Dpo4 or Cy5-labeled mutant (C31S, N70C) Dpo4 and then rapidly mixed with 100 μ M dTTP for increasing amounts of time. All fast reactions were performed using a rapid chemical-quench flow apparatus (Kintek). DNA products were then resolved on a denaturing 17% polyacrylamide gel, scanned using a Typhoon Trio (GE Healthcare), and quantitated with ImageQuant software (Molecular Dynamics). The product formation was then plotted against time (t) and the data were fit to Equation 5.

$$[\text{Product}] = A[1 - \exp(-k_{burst}t)] + k_{ss}t \quad (\text{Eq. 5})$$

where A represents the reaction amplitude, k_{burst} the single-turnover nucleotide incorporation rate constant, and k_{ss} the observed steady-state rate constant.

RESULTS

Design of a FRET System for Monitoring Dpo4 Interaction with DNA

To investigate the binding of Dpo4 at the single-molecule level, we adapted a previously designed FRET system to attach a Cy5 fluorophore to the Finger domain of Dpo4 (N70C) and a Cy3 fluorophore to each of the DNA substrates described in Figure 1.⁽²⁵⁾ Notably, Dpo4 is known to bind the blunt-end of DNA with a fivefold lower affinity as compared to the primer/template junction of a double-stranded DNA substrate but does not bind to single-stranded DNA.⁽⁴³⁾ Therefore, we added a non-complementary frayed end to each of the DNA substrates (Figure 1A) used in this article to eliminate off-target binding by Dpo4 which would have complicated data interpretation. Fluorescence spectra recorded upon titration of Cy5-labeled Dpo4 into a solution of Cy3-labeled undamaged DNA or 8-oxo-dG containing DNA (damaged DNA) indicate that the FRET probes are well-positioned to report on the binding of Dpo4 to DNA and that the fluorescent labels and 8-oxo-dG lesion did not affect the DNA binding affinity of Dpo4 (Figure 2), as reported previously.^(5, 26) To determine if the mutations or the label affected the polymerase activity of Dpo4, burst kinetic assays of Cy5-labeled Dpo4 were performed to obtain single-turnover nucleotide incorporation and steady-state rate constants. The rate constants for the labeled enzyme are similar to those of wild-type Dpo4 (Figure S3), indicating the mutations and fluorescent label did not alter the polymerase activity of Dpo4.

Investigation of Dpo4 in a Binary Complex with DNA by smFRET

To determine if the binding of Dpo4 to single DNA molecules is perturbed by the presence of a non-helix distorting oxidative lesion, 8-oxo-dG, we first conducted smFRET experiments with DNA substrates containing either a dG, or a 8-oxo-dG lesion at the templating position in the DNA (Figure 1). Overall, smFRET time trajectories for Dpo4 binding to either DNA substrate were similar, displaying transitions between three, non-zero FRET efficiency values as determined by visual inspection (Figure 1D). FRET efficiencies of >200 individual traces from each single-molecule experiment were collected and compiled into population distribution histograms (Figures 3A, 3B). The Gaussian peak fits of the histograms revealed subpopulations with FRET efficiencies centered at ~0.50 (low-FRET state), ~0.65 (mid-FRET state), and ~0.85 (high-FRET state). Consistently, smFRET investigations of other DNA polymerases, such as *E. coli* DNA polymerase I (Klenow fragment), and *S. solfataricus* Polymerase B1, have also revealed multiple FRET states which suggest various polymerase conformations while bound to DNA.^(30, 35, 40)

Interestingly, we observed that the oxidative lesion affected the population distributions represented in the histograms (Figure 3B). When bound to the undamaged DNA substrate, most FRET events (51%) were observed to be in the mid-FRET state. However, when an 8-oxo-dG lesion was present, most FRET events (50%) were observed to be in the low-FRET state. Unexpectedly, our data revealed a small, distinct distribution at a FRET efficiency higher than the two previously described.⁽³⁶⁾ This third state was observed regardless of the DNA substrate used. Given the inherent noise of smFRET data we chose to complement our manual data analysis with a probability-based Hidden Markov modelling analysis⁽⁴²⁾ to unbiasedly confirm the number of non-zero FRET efficiency states. The software idealized a subset of FRET trajectories (100) and converged on three, non-zero FRET states as expected from our visual inspection of the single-molecule trajectories (Figure 3A, B). Additional HMM analysis with the program TDP allowed for the construction of two-dimensional transition density plots (TDP) in which initial FRET efficiencies were plotted against final FRET efficiencies for every transition in the data sets (Figure S4). Importantly, if the smFRET data contain distinct, reproducible FRET values, then crosspeaks should develop in the TDPs. The corresponding plot in Figure S4 clearly shows crosspeaks representing transitions amongst all three states for each of the DNA substrates evaluated. Prominent density in the plots at the high→mid, mid→high, high→low, low→high, mid→low, and low→mid transition areas indicates that Dpo4 was able to freely convert between the three FRET states. Additionally, our TDP analysis gave the shuttling rates for the transitions between the FRET states. Overall, shuttling rate between all states for Dpo4 binding to either DNA substrate were similar (Table 1).

To further investigate the response of Dpo4 to undamaged or damaged DNA, a dwell time analysis of the single-molecule binding traces was performed to yield information on the dissociation kinetics of the binary complexes. Dwell time histograms and survivor functions for this analysis are located in Figures S5A and S6A. The survivor functions were best fit to a double exponential decay equation indicating that binding events for the binary complex (Dpo4•DNA) vary in stability (Table 2 and Figure S7). Notably, a modest decrease in the fast phase rate constant ($k_{\text{off},1}$) and increase in the slow phase amplitude (A_2) for Dpo4 dissociation from the damaged DNA substrate relative to the undamaged one suggest a

modest increase in the complex stability of Dpo4•DNA caused by the presence of the oxidative lesion.

Effect of dNTPs on Dpo4 Binding to Undamaged or Damaged DNA

smFRET experiments were performed to probe whether or not the presence of an incoming nucleotide affected the binding of Dpo4 to DNA. Notably, the addition of correct or incorrect dNTP (6 mM) eliminated the high-FRET state that was observed for the binary complex Dpo4•DNA with either the undamaged or damaged DNA substrate (Figures 3C, 4, S8, S9). With correct dCTP, smFRET time trajectories clearly displayed a single, sustained FRET state and the population histograms contain one low-FRET distribution (~0.5) (Figure 3C and S8). Further, HMM and TDP analysis (Figure S4) indicate negligible transitions between non-zero FRET states.

Dwell time analysis for Dpo4 binding to either undamaged or damaged DNA in the presence of a correct incoming nucleotide revealed slower (2- to 3-fold) dissociation kinetics compared to the binary complex (Dpo4•DNA) indicating that the correct dNTP stabilizes the ternary complex (Dpo4•DNA•dNTP) (Table 2). Dwell time histograms and survivor functions for this analysis are located in Figures S5B and S6B. Remarkably, the correct nucleotide seemed to have a greater stabilizing effect for Dpo4 binding to the damaged DNA substrate as observed through slower dissociation rates (Table 2).

Experiments to observe the effect of an incorrect incoming nucleotide on the conformations and kinetics of Dpo4 binding to undamaged or damaged DNA substrates were performed to uncover the mechanism of nucleotide selection by Dpo4. smFRET trajectories of Dpo4 binding in the presence of each of the three incorrect nucleotides display short-lived FRET events with two primary FRET efficiencies (Figure S9). Population histograms further revealed a bimodal distribution (Figure 4). Despite the high concentrations of incorrect nucleotide used in each experiment (6 mM), the bimodal distribution may be a result of an inability fully saturate the enzyme.⁽⁴⁴⁾ Dwell time analysis confirmed that Dpo4 binding events to either DNA substrate were transient with fast dissociation kinetics (Table 2, Figures S5 and S6). However, dwell times for Dpo4 binding to the damaged versus to the undamaged DNA substrate in the presence of an incorrect nucleotide were marginally longer lived. While the dwell time histograms for the dissociation of Dpo4 from undamaged or damaged DNA in the presence of incorrect nucleotide fit well to the single exponential decay equation (Equation 3), we cannot completely exclude the possibility of longer time scale processes.

DISCUSSION

Through smFRET investigation, we demonstrated that the binding of Dpo4 to DNA is a complex process. The smFRET trajectories and FRET population histograms for this polymerase binding to an undamaged or a damaged DNA substrate (Figures 1A) revealed three FRET states (Figure 3A and 3B). Interestingly, our previous stopped-flow FRET studies^(25–27) have revealed that Dpo4 and DNA forms an initial binary complex (Complex I), which transitions into a ternary complex (Complex II) after an incoming dNTP stimulates the rapid translocation of Dpo4 along the DNA axis by one base pair in order to empty the

space within the active site for nucleotide binding (Scheme 1A). Subsequently, synchronized domain motions of Dpo4 help to tighten its “grip” on both the DNA and nucleotide, leading to the formation of Complex III. Prior to phosphodiester bond formation, Complex IV is formed when Dpo4 undergoes the rate-limiting active site rearrangement to reposition key active site residues and properly align all substrates for catalysis (Scheme 1A).^(44, 45) Notably, the binary and ternary crystal structures of Dpo4^(24, 29) represent Complexes I and IV, respectively, but do not inform on Complexes II and III (Scheme 1A). Surprisingly, the primer/template junction base pair in the binary crystal structure occupies the same space as the nascent base pair in the ternary structure,⁽²⁹⁾ suggesting DNA translocation by one base pair during nucleotide binding. Coupled with the aforementioned stopped-flow FRET^(25–27) and X-ray crystallographic studies^(24, 29), we conclude that the mid-FRET state shown in Figures 3A and 3B likely represents Complex I while the low-FRET state corresponds to the conformation(s) of Dpo4 in Complex III and/or IV wherein DNA has translocated by one base pair and Dpo4 has undergone synchronized domain motions. More importantly, the low-FRET state suggests that Dpo4 might have sampled the conformations in Complexes II–IV throughout the DNA binding process (Dpo4•DNA) in our smFRET experiments, even in the absence of an incoming nucleotide. However, as Complex II is a transient species, existing briefly during the nucleotide binding process, the low-FRET state observed during DNA binding by Dpo4 can only represent Complexes III or IV. Interestingly, the addition of correct nucleotide stabilized the polymerase in the low FRET efficiency state representing Complexes III and IV (Figures 3C and S8). Notably, Complexes III and IV cannot be distinguished by FRET as they differ by small active site rearrangements which do not affect the distance between the FRET pair in our system (Figure 1).

Consistently, a recently published smFRET study of the binding of Dpo4 to undamaged DNA has revealed two similar FRET states which are correlated to a pre-insertion and an insertion site on the DNA substrate.⁽³⁶⁾ The pre-insertion site is consistent with our assignment of Complex I. However, those authors have not considered that the low-FRET state represents Complex III and/or IV. In addition, they concluded that the transition rates ($1.1\text{--}2.7\text{ s}^{-1}$) between the two FRET states correspond to the rate of DNA translocation.⁽³⁶⁾ Although our shuttling rates for Dpo4 transitioning between the mid- and low-FRET states with undamaged DNA ($2.8\text{--}3.3\text{ s}^{-1}$, Table 1) are similar, these rates are too slow to account for the rapid translocation of Dpo4 along DNA. In fact, the translocation was too fast to be accurately measured by our stopped-flow FRET studies at 20 °C, implying a rate of $>150\text{ s}^{-1}$.^(25, 26) Recent single-molecule investigations^(46–48) also suggest that the translocation rate is too fast to be resolved at the frame rates of conventional smFRET cameras (80 ms for the previous study⁽³⁶⁾ and 100 ms for this study). Accurate rate estimation requires high temporal and spatial resolution such as that obtained in a recent study of phi29 polymerase where single-molecule nanopore technology was used to estimate a DNA translocation rate of $>250\text{ s}^{-1}$ at 21 °C.^(46–48) Interestingly, the synchronized domain motions of Dpo4 in Step 3 (Scheme 1A) occur at rates of $\sim 10\text{ s}^{-1}$ which are reasonably comparable to our shuttling rates (Table 1).^(25–27) Taken together, the shuttling rates obtained here and in the previous study⁽³⁶⁾ for Dpo4 transitioning between the mid- and low-FRET states are not specifically reporting on the DNA translocation event, but rather on concomitant domain motions as observed through the Cy5-labeled Finger domain. Thus, our single-molecule investigation

yielded results which are consistent with those from ensemble studies^(25–27) and unveiled a dynamic conformational equilibrium in the domains of Dpo4 that accompanied DNA translocation.

The high-FRET state (0.87) detected in Figure 3A was unexpected but confirmed through repeated experiments and unbiased HMM analysis. Analysis of our single molecule data indicates that Dpo4 can bind directly in this high-FRET state as well as access it through either the low-FRET or mid-FRET state (Scheme 1B). Notably, the absence of this high-FRET state in the previous smFRET study of Dpo4⁽³⁶⁾ likely stems from the different fluorophore labelling positions selected for the polymerase and DNA substrates in this work. Although more work is required to characterize this high-FRET state, we can speculate on its identity and significance here. Superposition of the apo and the binary (Dpo4•DNA) crystal structures has revealed large structural changes, especially a dramatic 131° rotation of the LF domain relative to the polymerase core, accompanying DNA binding.⁽²⁹⁾ The conformational flexibility of the LF domain is facilitated by a highly flexible, 14 amino acid residue linker connecting the LF and Thumb domains of Dpo4. This flexibility is further illustrated in the crystal structure of Dpo4 in complex with a subunit of heterotrimeric proliferating cell nuclear antigen (PCNA) where the LF domain exists in an extended conformation distinct from that in the apo and binary structures.⁽⁴⁹⁾ Furthermore, our recent computational analysis of Dpo4 binding to DNA suggests that the LF domain and linker are intimately involved in the complex, multi-step processes the polymerase utilizes to recognize and bind DNA.⁽⁵⁰⁾ Given the inherent plasticity of the LF domain and linker as well as the conformational equilibria between multiple binding states (Schemes 1A, 1B), we hypothesize that the observed high-FRET state is related to an alternate DNA binding conformation of Dpo4 facilitated by both the dynamic LF domain and the flexible linker (Scheme 2A). Interestingly, this high-FRET state is eliminated in the presence of dNTPs, suggesting that this binding mode may be non-productive and the presence of the nucleotide stimulates its conversion into a catalytically productive conformation at the primer/template junction. Alternatively, Dpo4 may sample other non-productive conformations, such as the polymerase binding to DNA in an inverted conformation which would shorten the distance between the Cy5 in the Finger domain and the Cy3 in the DNA substrate (Scheme 2B). Consistently, multiple binding orientations for an enzyme bound to a nucleic acid substrate have been previously observed by smFRET.^(51, 52) Research is currently under way to explicitly characterize this high-FRET state.

By introducing an incoming nucleotide to the binary complex of Dpo4 with undamaged or damaged DNA, we revealed the stringent nucleotide selectivity of Dpo4 through smFRET experiments. Correct dNTP stabilized the binding of Dpo4•DNA as revealed by the complete shift of its FRET distributions to the low-FRET state (Complexes III and IV in Scheme 1A) (Figures 3C and S8) and more than 2-fold slower dissociation kinetics of Dpo4 from undamaged or damaged DNA (Table 2). Contrastingly, upon the introduction of an incorrect dNTP, we observed a bimodal population distribution as a result of incomplete population shifts to the low-FRET state (Figure 4). Our dwell time analysis revealed that Dpo4 remained bound to DNA 5- to 10-fold longer in the presence of a correct over an incorrect nucleotide (Table 2). Additionally, observed biphasic decay kinetics in the presence of correct dCTP (Figures S5B and S6B, Table 2) suggest the existence of the loose (E

'•DNA•dNTP) and tight (E'' •DNA•dNTP) ternary complexes, which is consistent with our previously established kinetic mechanism (Scheme 1C).^(44, 53) Interestingly, only fast, single exponential decay kinetics were observed in the presence of an incorrect nucleotide, suggesting the preferred collapse of the loose ternary complex (Figures S5 and S6, Table 2). Thus, our single-molecule analysis indicates that correct nucleotide binding allowed the formation of the catalytically competent, tight complex (E'' •DNA•dNTP). Interestingly, two competition experiments were conducted wherein the Dpo4•DNA complex was incubated with equal concentrations of either the three incorrect dNTPs or all four dNTPs. In both experiments, the FRET population distribution was bimodal. However, more FRET events were in the low-FRET state in the presence over absence of correct dCTP (83% versus 58%, Figure S10). Taken together, these smFRET experiments demonstrated that Dpo4 was able to differentiate between correct versus incorrect dNTPs during nucleotide binding.

Previously, our ³²P-based kinetic assays monitoring the 8-oxo-dG bypass have revealed that Dpo4 exhibits surprisingly high fidelity and efficiency. In fact, the efficiency of correct incorporation opposite 8-oxo-dG is higher than that opposite the undamaged templating dG.⁽⁵⁾ Notably, our smFRET time trajectories are similar between Dpo4 binding to undamaged versus damaged DNA (Dpo4•DNA) but a smaller low-FRET state population was observed with the former (35% versus 50%, Figures 3A and 3B). More importantly, the dissociation kinetics of ternary complexes with correct dCTP show a larger population of slower dissociation events from the damaged over undamaged DNA substrates (69.8% versus 53.1%, Table 2). In the presence of either correct or incorrect nucleotides, ternary complex dissociation is noticeably slower from the damaged than the undamaged DNA substrate (Table 2). Consistently, the ternary crystal structure of Dpo4 with DNA containing a templating 8-oxo-dG and an incoming dCTP shows an auxiliary hydrogen bond and an ion-dipole pair between Arg332 and 8-oxo-dG.⁽²⁴⁾ Overall, our smFRET analysis indicates that Dpo4 preferentially binds DNA containing 8-oxo-dG through additional stabilizing interactions with the lesion. Interestingly, it has been previously hypothesized that Y-family DNA polymerases derive their lesion bypass specificity through unique active site residues which are optimal for their interactions with the templating lesion(s).^(54, 55) Consistently, our smFRET experiments help to explain the preference of Dpo4 for bypassing 8-oxo-dG which constantly challenges the *Sulfolobus solfataricus* replication machinery *in vivo*.⁽⁵⁶⁾

Notably, comparison of FRET efficiency histograms with damaged DNA (Figure 4) shows no preference of Dpo4 for any particular incorrect nucleotide, including dATP despite its capability of forming a Hoogsteen base pair with 8-oxo-dG. Consistently, ternary crystal structures show that Arg332 stabilizes the *anti*- over *syn*-conformation of 8-oxo-dG at the active site of Dpo4 through the hydrogen bond and the ion-dipole pair discussed above.^(10, 24)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

8-oxo-dG	8-oxo-7,8-dihydro-2'-deoxyguanine
Dpo4	DNA Polymerase IV of <i>Sulfolobus solfataricus</i>
smFRET	single-molecule Förster resonance energy transfer
TLS	translesion DNA synthesis
TDP	transition density plot
HMM	hidden Markov modeling
dNTPs	deoxynucleotide triphosphates
LF	Little Finger

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Figure 1. Single-molecule FRET analysis of Dpo4 binding to DNA

(A) DNA substrate used in single-molecule experiments. The primer is biotinylated at the 5' end (denoted "B") to facilitate surface immobilization. "T" denotes Cy3 attached to 5-C6-amino-2'-deoxythymidine. "G" represents 8-oxo-7,8-dihydro-2'-deoxyguanine (8-oxo-dG) or undamaged dG. (B) The binary complex of Dpo4 and DNA accessed through PDB code 2RDJ. The green star represents a Cy3 donor label on the DNA substrate. The red star signifies the site of Cy5 acceptor labeling in the Finger domain (N70C) of Dpo4. (C) Chemical structure of 8-oxo-dG with chemical modifications at the C8 and N7 positions of guanine circled in red. (D) Representative single-molecule fluorescence trajectory for Dpo4 binding to the DNA substrate containing 8-oxo-dG. Donor and acceptor fluorescence signals are shown in green and red, respectively.

Figure 2. Ensemble FRET Analysis of Dpo4 Binding

(A) Example fluorescence titration. Cy3-DNA (25 nM) was excited at 532 nm and fluorescence emission spectra were recorded at varying concentrations of Cy5-Dpo4 N70C ranging from 0 nM (*black*) to 180 nM (*pink*). (B) Change in donor quantum yield (ϕ_{Donor}) for the undamaged (●) or 8-oxo-dG modified (■) DNA substrates upon titration with Dpo4. The curves were fit to Eq. 1 to yield a K_d of 11 ± 1 nM for Dpo4 binding to undamaged DNA and a K_d of 10 ± 1 nM for Dpo4 binding to the 8-oxo-dG modified DNA.

Figure 3. FRET from Dpo4 binding to single DNA molecules

FRET efficiency histograms (left) and representative smFRET trajectories (right) of Dpo4 binding to (A) undamaged DNA (B) 8-oxo-dG modified DNA and (C) 8-oxo-dG modified DNA with 6 mM of correct nucleotide (dCTP). On the left, individual Gaussian peak fits for each state are shown by the dashed black lines with respective population percentage indicated in red. The sum of the individual Gaussians is shown as the solid black line. On the right, representative FRET trajectories are shown in blue and the black lines depict HMM fits to the data.

Figure 4. FRET Histograms of Dpo4 binding DNA with incorrect dNTP

(A) Population distribution histograms generated from multiple time trajectories of Dpo4 binding to undamaged DNA (top) or 8-oxo-dG modified DNA (bottom) in the presence of 6 mM of either dTTP (A, D), dGTP (B, E), or dATP (C, F). Individual Gaussian peak fits for each state are shown by the dashed black lines with respective population percentage indicated in red. The sum of the individual Gaussians are shown as the solid black line in each.

Scheme 1. Proposed Mechanism of Binary and Ternary Complex Formation

(A) Previous proposed mechanism of nucleotide incorporation. E, E', and E'' are different forms of the enzyme. Steps after 4 were not shown for clarity of discussion. (B) Binary complex formation. $[E \cdot DNA]^L$, $[E \cdot DNA]^M$, and $[E \cdot DNA]^H$ refer to low-, mid-, and high-FRET binary complexes, respectively. (C) Ternary complex mechanism. $[E' \cdot DNA \cdot dNTP]^L$ and $[E'' \cdot DNA \cdot dNTP]^L$ refer to two different forms of the ternary complex.

Scheme 2. Conformational sampling of the high-FRET state

(A) The LF domain of Dpo4 has been shown to adopt multiple structural conformations. The high-FRET state may be an extended conformation of the LF domain. Proximity between fluorophores increases during transition from a compact binary complex to an extended conformation. The LF binds DNA autonomously allowing the core domains to sample conformational states and DNA surface area as facilitated by the flexible linker region (B) Dpo4 may adopt a nonproductive conformation in which the polymerase is incorrectly oriented on the DNA resulting in high-FRET as the proximity between Cy5 in the Finger domain and Cy3 on the substrate increases.

Table 1

Shuttling Rates from Transition Density Plot

Transition	Undamaged Substrate (s^{-1})	8-oxo-dG modified Substrate (s^{-1})
L→M	2.8 ± 0.7	3.2 ± 0.8
L→H	1.5 ± 0.4	1.0 ± 0.4
M→L	3.3 ± 0.8	4 ± 2
M→H	1.8 ± 0.7	1.8 ± 0.5
H→L	1.8 ± 0.4	5 ± 2
H→M	1.9 ± 0.5	3 ± 1

L = Low-FRET, M = Mid-FRET, H = High-FRET

Table 2**Dwell Time Analysis of Dpo4 Binding to DNA**

Average Binding Time (s)		A_1	$k_{off,1}$ (s ⁻¹)	A_2	$k_{off,2}$ (s ⁻¹)
Undamaged DNA Substrate					
DNA	1.3	0.51 ± 0.02 (50.5%)	2.5 ± 0.1	0.50 ± 0.02 (49.5%)	0.50 ± 0.01
DNA + dCTP	2.7	0.46 ± 0.02 (46.9%)	6.3 ± 0.5	0.52 ± 0.01 (53.1%)	0.215 ± 0.005
DNA + dATP	0.3	1.01 ± 0.01	5.0 ± 0.1	-	-
DNA + dGTP	0.2	1.02 ± 0.02	6.2 ± 0.2	-	-
DNA + dTTP	0.3	1.02 ± 0.02	5.7 ± 0.2	-	-
Damaged DNA Substrate					
DNA	1.6	0.38 ± 0.04 (38.8%)	1.7 ± 0.01	0.60 ± 0.04 (61.2%)	0.46 ± 0.02
DNA + dCTP	4.2	0.29 ± 0.01 (30.2%)	1.16 ± 0.08	0.67 ± 0.01 (69.8%)	0.185 ± 0.003
DNA + dATP	0.8	0.97 ± 0.02	1.38 ± 0.04	-	-
DNA + dGTP	0.6	1.04 ± 0.02	2.2 ± 0.07	-	-
DNA + dTTP	0.9	0.92 ± 0.02	1.13 ± 0.03	-	-