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Advances in Structural and Single-Molecule Methods for Investigating DNA Lesion Bypass and Repair Polymerases

Austin T. Raper[†], Andrew J. Reed[†], Varun V. Gadkari, and Zucui Suo^{*}

Department of Chemistry and Biochemistry and The Ohio State Biochemistry Program, The Ohio State University, Columbus, Ohio 43210, United States

Abstract

Innovative advances in X-ray crystallography and single-molecule biophysics have yielded unprecedented insight into the mechanisms of DNA lesion bypass and damage repair. Time-dependent X-ray crystallography has been successfully applied to view the bypass of 8-oxo-7,8-dihydro-2'-deoxyguanine (8-oxoG), a major oxidative DNA lesion, and the incorporation of the triphosphate form, 8-oxo-dGTP, catalyzed by human DNA polymerase β . Significant findings of these studies are highlighted here, and their contributions to the current mechanistic understanding of mutagenic translesion DNA synthesis (TLS) and base excision repair are discussed. In addition, single-molecule Forster resonance energy transfer (smFRET) techniques have recently been adapted to investigate nucleotide binding and incorporation opposite undamaged dG and 8-oxoG by *Sulfolobus solfataricus* DNA polymerase IV (Dpo4), a model Y-family DNA polymerase. The mechanistic response of Dpo4 to a DNA lesion and the complex smFRET technique are described here. In this perspective, we also describe how time-dependent X-ray crystallography and smFRET can be used to achieve the spatial and temporal resolutions necessary to answer some of the mechanistic questions that remain in the fields of TLS and DNA damage repair.

Graphical abstract

INTRODUCTION

Over the last few decades, more emphasis has been placed on structural evidence of biochemical events than ever before. Although many enzyme mechanisms can be inferred

^{*}Corresponding Author Tel: +1 614 6883706. Fax: +1 614 2926773. suo.3@osu.edu.

[†]A.T.R. and A.J.R. contributed equally to this work.

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from the results yielded through typical biochemical methods, convincing structural evidence is often necessary to validate a particular model. For example, by solving the crystal structures of the apo form, the binary complex (E·DNA), the precatalytic ternary complex (E·DNA·dNTP), and the product ternary complex (E·DNA·pyrophosphate) of numerous DNA polymerases across all six phylogenetic families (A, B, C, D, X, and Y),¹ which function in DNA replication, DNA damage repair, and translesion DNA synthesis (TLS),^{2–19} the mechanism of DNA synthesis at the atomic-level was largely determined. Accordingly, more than 1,000 such crystal structures of DNA polymerases provide valuable information on the binding and positioning of substrates (DNA and dNTP) and products (DNA and pyrophosphate), the positioning and coordination of divalent metal ions, the binding conformations of active site residues, and putative protein conformational changes. Although spatially informative, traditional structural techniques are limited to static snapshots of stable states; therefore, certain mechanistic details of the transition between reactant and product complexes of polymerase-catalyzed nucleotide incorporation may still be ambiguous or unidentified.

For the disadvantages of conventional X-ray crystallography to be overcome, time-dependent X-ray crystallography was recently developed and applied to follow polymerase-catalyzed reactions.^{20–25} This relatively inexpensive and simple to execute technique has allowed several research laboratories to temporally capture the structures of reaction intermediates at atomic level resolutions.^{20–25} As this elegant technique will likely change the research landscape for structurally characterizing biological processes such as DNA replication, DNA damage repair, and TLS, we will highlight several notable time-dependent X-ray crystallographic studies of DNA polymerases and give our opinions for new research directions in this perspective. Although useful, time-dependent X-ray crystallography is limited to enzyme reactions that can be readily activated in crystallo and may only illuminate specific steps of a multistep enzyme mechanism. For DNA polymerases, this has been limited to the chemistry step of nucleotide incorporation (Scheme 1, step 6). Therefore, various mechanistic events, such as DNA binding, DNA translocation, nucleotide binding, protein conformational changes (Scheme 1, steps 1, 4, 5, 7, and 8), and product release (Scheme 1, step 9) cannot be observed. Furthermore, although time-dependent crystallography is possible through slowed enzyme catalysis in crystallo, any measured rates cannot be reliably compared to solution-based kinetics. Thus, the high-spatial resolution afforded by this technique comes at the cost of temporal resolution.

Consequently, a more versatile approach capable of accessing faster, more relevant time scales and multiple enzymatic steps is necessary to fully probe the catalytic mechanism. In recent years, advances in microscope optics, high-resolution cameras, and state-of-the-art data acquisition and analysis software have spurred the advent and rapid growth of single-molecule methodologies by which to study biological processes in vitro. One particularly informative method, single-molecule Forster resonance energy transfer (smFRET),²⁶ reports on distance changes between site-specifically placed donor and acceptor fluorophores covalently linked to nucleic acid substrates or enzymes of interest. In this way, the solution behavior of individual molecules can be tracked in real-time to give unparalleled insight into complex enzyme mechanisms as well as protein–protein and protein–DNA interactions. Ensemble steady-state and presteady-state kinetic methodologies,²⁷ although very

informative and valuable, can only be utilized to report on the collective or average behavior of all molecules during a reaction.²⁶ As a result of this limitation, information on critical minor species or subpopulations of molecules during an enzyme-catalyzed reaction that may perform distinct functions and/or exist in different conformational states is lost. Furthermore, by following the reaction trajectories of individual molecules, smFRET has the remarkable ability to show the order of events for a given enzyme-catalyzed reaction without the need for synchronization, which is difficult or impossible for some systems. Thus, smFRET allows for simultaneous spatial and temporal characterization of an enzyme-catalyzed reaction. We have recently applied this technique to study *Sulfolobus solfataricus* DNA polymerase IV (Dpo4), a model Y-family DNA polymerase,²⁸ as it encountered and bypassed an 8-oxo-7,8-dihydro-2'-deoxyguanine (8-oxoG) lesion, a major oxidative DNA lesion, on a DNA template during DNA synthesis.²⁹ Structural studies have established that 8-oxoG does not significantly distort the structure of DNA; however, its mutagenicity results from its ability to readily form two possible base pairing conformations within the polymerase active site.^{30–32} Furthermore, published studies have shown that 8-oxoG forms specific interactions with polymerase active site residues to directly affect lesion bypass activities.^{33–38}

Because of the wealth of data that single-molecule techniques can provide, we will give a more thorough description of our recent article in this perspective, as well as comment on future applications of this methodology to the study of DNA replication, TLS, and DNA damage repair. Previously, our laboratory has published many articles on the bypass of a variety of DNA lesions catalyzed by X- and Y-family DNA polymerases.^{5,24,26,38–54} Because most of these articles have been reviewed recently,²⁷ here we mainly discuss our work on 8-oxoG bypass catalyzed by Dpo4.^{29,39}

TIME-RESOLVED X-RAY CRYSTALLOGRAPHY

DNA polymerases bind DNA and dNTP sequentially in addition to binding and coordinating divalent metal ions (e.g., Mg^{2+} or Mn^{2+}) to three conserved carboxylate residues at the active site to catalyze a nucleotidyl transfer reaction. Beginning in 2005, our laboratory attempted to perform time-dependent nucleotide incorporation in the crystals of Dpo4 through soaking its binary complex crystals (E-DNA) with an incoming correct or incorrect dNTP and Mg^{2+} for various amounts of time. However, this method resulted in the deterioration of crystal morphology and prevented us from obtaining high-resolution structures of reaction intermediate states. Instead, we collaborated with the laboratory of Paul Carey and utilized low-resolution single-crystal Raman crystallography to obtain useful mechanistic information on nucleotide incorporation and protein conformational changes in crystallo.⁴⁰ In contrast to our misfortune, the laboratory of Katsuhiko Murakami employed similar time-dependent soak-trigger-freeze X-ray crystallography and solved the high-resolution crystal structures of precatalytic complex and two reaction intermediate states of bacteriophage N4 RNA polymerase.²² Their structures reveal that the nucleotide-binding divalent metal ion at the B-site binds first followed by the catalytic divalent metal ion at the A-site. Following A-site divalent metal ion binding, a phosphodiester bond was formed between the two bound rNTPs producing a 2-mer RNA transcript at the active site of bacteriophage N4 RNA polymerase, after which the A-site divalent metal ion quickly

dissociates.²² However, they did not observe any reaction intermediates for bond formation or the third divalent metal ion bound at the C-site (see below)

Concurrently, the laboratory of Wei Yang pioneered a new method for time-dependent X-ray crystallographic studies of polymerases.²¹ Rather than the aforementioned soak-trigger-freeze method where nucleotide and catalytic divalent metal ions were soaked simultaneously, their methodology takes advantage of the fact that the binding of Ca^{2+} at a polymerase active site allows for proper binding and positioning of DNA and nucleotide but does not support catalysis.²¹ First, a precatalytic ternary complex (Scheme 1, E "·DNA-dNTP) that has gone through all elementary steps in a kinetic mechanism (Scheme 1, steps 1–5) prior to phosphodiester bond formation (step 6)^{29,41–43} was crystallized in the presence of noncatalytic Ca^{2+} . The Yang laboratory then initiated phosphodiester bond formation in crystallo via Ca^{2+} to Mg^{2+} ion exchange. This step was accomplished by removing crystals of the precatalytic ternary complex from the crystallization solution and transferring them to a cryoprotectant solution containing an excess of catalytic Mg^{2+} for a variable amount of time (seconds to minutes) before freeze-quenching the reaction in liquid nitrogen (Figure 1A). This methodology affords visualization of reaction intermediates in which mechanistic intricacies, such as chemical bond formation and breakage, steric inversion of the $\text{S}_{\text{N}}2$ reaction center, changes in side chain rotamer conformations, DNA and dNTP binding conformations, and divalent metal ion coordination can be directly observed.²¹ More significantly, the Yang laboratory unprecedentedly observed the appearance of a third Mg^{2+} ion during phosphodiester bond formation (Scheme 1, step 6).²¹

Very recently, the Yang methodology was successfully adapted by Samuel Wilson's group and our laboratory^{23–25} to investigate phosphodiester bond formation catalyzed by full-length human DNA polymerase β (hPol β), an X-family polymerase. Wilson's group employed time-dependent X-ray crystallography to watch how hPol β incorporated undamaged correct and incorrect dNTPs²³ as well as a damaged nucleotide (8-oxo-dGTP),²⁴ and we were the first to view both faithful (Figure 1B–D) and unfaithful TLS across a DNA lesion (8-oxoG).²⁵ Notably, the mutagenic bypass of the major oxidative DNA lesion 8-oxoG during base excision repair (BER) in vivo could lead to cancer formation.^{44–47}

Rotational freedom about the glycosidic bond (anti/syn conformations) and the additional hydrogen bonding capabilities of 8-oxo-dGTP and 8-oxoG on the Hoogsteen face (oxidation of C8 and protonation of N7) result in the dual coding potential of the oxidized nucleotide and the damaged templating base (Figure 2). Therefore, *anti*-8-oxo-dGTP and *anti*-8-oxoG can Watson–Crick base pair with cytosine (Figure 2A and C), and *syn*-8-oxo-dGTP and *syn*-8-oxoG can Hoogsteen base pair with adenosine (Figure 2B and D). In the time-dependent studies, both Wilson and our laboratories identified key side chain interactions that support the previously reported dNTP and templating base preference for 8-oxoG bypass and 8-oxo-dGTP incorporation, respectively (Figure 2).⁴⁸ Notably, R283 of the thumb domain acts as a negative selection factor against 8-oxo-dGTP incorporation opposite dC through electrostatic repulsion between the side chain of R283 and the N2 position of 8-oxo-dGTP (Figure 2C).²⁴ R283 was also shown to stabilize the *syn*-conformation of a templating 8-oxoG during dATP misincorporation (Figure 2B).²⁵ Interestingly, this structural information is consistent with our previous kinetic characterization of 8-oxoG

bypass or 8-oxo-dGTP incorporation by hPol β and suggests that R283 modulates the mutagenicity of these processes.^{24,25,48}

In addition, in the structures where partial dCTP (Figure 1C) or dATP incorporation opposite 8-oxoG was observed, the primer 3'-OH moves 0.8–1.3 Å relative to its position in the precatalytic ternary structures and initiates the nucleophilic attack on the P α of the incoming dNTP, resulting in a 1.0–1.1 Å shift of the P α toward the 3'-OH and the steric inversion of the nonbridging oxygen atoms as expected for an S_N2 reaction.²⁵ Furthermore, key details for the postcatalytic process were identified including a stacking interaction between the newly incorporated nucleotide and the templating 8-oxoG, suggesting a role in preventing successive nucleotide incorporation and strand displacement on the nicked DNA product.²⁵

More interestingly, as with the first time-dependent crystallography study of phosphoryl transfer by hPol η ,²¹ all three studies on hPol β revealed three divalent metal ions (Mg²⁺ or Mn²⁺) at the polymerase active site during phosphodiester bond formation.^{23–25} The third divalent metal ion was unambiguously detected when Ca²⁺ was exchanged in crystallo with Mn²⁺,^{22,24} which yielded anomalous scattering and suggests that polymerases use a “three-metal-ion mechanism” (Figure 1C,D). These time-dependent X-ray crystallographic studies^{20–25} clearly established that the binding of the divalent metal ions before phosphodiester bond formation follows the order of the B-, A-, and C-site, whereas their dissociation order after the chemistry step is the A-, C-, and B-site. These results challenge the long-standing “two-metal-ion mechanism” for the nucleotidyl transfer reaction proposed by Lauren Beese and Tom Steitz nearly two decades ago.⁴⁹ The Steitz laboratory solved the first structures of the Klenow fragment of *E. coli* DNA polymerase I and discovered that the exonuclease activity appears to require two Mg²⁺ ions,⁴⁹ and therefore, it was hypothesized that all phosphoryl transfer reactions including polymerase-catalyzed nucleotide incorporation^{50,51} likely follow a two-divalent metalion mechanism. Their results have since been supported by hundreds of precatalytic ternary structures of other DNA polymerases^{9,17,51–54} in which nucleotide and two divalent metal ions are bound at the polymerase active site. In the “two-metal-ion mechanism”, the A- and B-site divalent metal ions are associated with the deprotonation of the primer terminus and the binding of incoming nucleotide, respectively.

However, through the time-dependent X-ray crystallography studies,^{21,23–25} the observed third Mg²⁺ ion at the C-site appears in a position bridging the α - and β -phosphates of the incoming nucleotide following reaction initiation (Figure 1C) and persisting through completion of nucleotide incorporation (Figure 1D) with its role in catalysis still debated and several competing hypotheses proposed. The positioning and timing of the third divalent metal ion have led Wilson's group to initially hypothesize a role in the reverse reaction, pyrophosphorolysis,²³ whereas others^{20,21,24,55} and our laboratory²⁵ have suggested the third metal ion is involved in transition state stabilization and may aid in pyrophosphate release (Figure 1C,D). In any case, the necessity of a third divalent metal ion for catalysis is hard to discredit with five recent structural studies^{20,21,23–25} and two computational analyses^{56,57} that detail its presence.

Although further biochemical or biophysical characterization is necessary to elucidate the role of the third divalent metal ion, it does not coordinate protein residues, and therefore, it is difficult to definitively assay its role in catalysis through simple mutational studies. However, if the third divalent metal ion serves a catalytic role in the forward reaction, then it should also participate in the reverse reaction. Consistently, the Wilson laboratory was able to crystallize the ground state of the reverse pyrophosphorolysis reaction with the third divalent metal ion bound but did not capture any reaction state structures (presumably because the reverse reaction is unfavorable), which lead them to hypothesize its importance in either pyrophosphate release or pyrophosphorolysis.²³ The Yang group was the first to attempt to assay the functional role of the third divalent metal ion by measuring metal ion saturation in crystallo and then correlating this with the amount of divalent metal ion necessary for nucleotidyl transfer in solution.²⁰ Although this was a reasonable approach, it would be perhaps more convincing to determine its functional role through techniques other than time-dependent X-ray crystallography. For example, our laboratory is utilizing electroparamagnetic resonance (EPR) spectroscopy to investigate the functional roles of divalent metal ions on the activity of hPol β .

Notably, the above mechanistic details as well as others presented in the time-dependent X-ray crystallographic studies^{20,21,23–25} are unobservable with conventional X-ray crystallography. It will be interesting to see if some of the new mechanistic aspects, such as the “three-metal-ion mechanism”, will hold true for other DNA polymerases, especially those in the A–D families as well as reverse transcriptases. It will also be exciting to watch how other types of DNA lesions, e.g., bulky single-base lesions, double-base lesions, and tandem lesions, are accommodated and bypassed by the X- and Y-family DNA polymerases. Furthermore, although the mechanistic steps pertaining to the reverse conformational change, pyrophosphate release, and DNA translocation or release (Scheme 1, steps 7–9) have remained elusive to structural characterization, time-dependent X-ray crystallography has the potential to probe these processes to further define the polymerase-catalyzed mechanism of nucleotide incorporation (Scheme 1).

SINGLE-MOLECULE FÖRSTER RESONANCE ENERGY TRANSFER

smFRET can be applied to study distance changes between donor and acceptor fluorescent dyes covalently attached to one or more interacting biomolecules. The donor and acceptor fluorophores must meet several photophysical criteria²⁶ and be positioned such that the interfluorophore distance is limited to $R = R_0 \pm 0.5R_0$, where R is the interfluorophore distance and R_0 is the calculated Forster radius, which is unique to the selected dye pair and depends on several important parameters²⁶ not discussed here. When properly designed, a FRET system can be very sensitive to distance changes, yielding low-resolution structural information for distance changes occurring between the donor and acceptor within 10–75 Å.
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For applying this technique for the monitoring of single molecules, especially DNA polymerases, one very popular strategy involves the surface immobilization of a DNA substrate that is chemically modified to contain a donor fluorophore and 5′- or 3′-biotin group (Figure 3A). Quartz slides and glass coverslips are chemically functionalized and

passivated with a lawn of polyethylene glycol molecules (some of which are modified with biotin bound by NeutrAvidin) as well as sandwiched together and sealed to form a reaction chamber. The modified DNA is then flowed into the chamber and immobilized via the very tight biotin–NeutrAvidin interaction. Prism-based total internal reflection fluorescence (TIRF)²⁶ is then utilized to greatly reduce fluorescent background to achieve signal-to-noise ratios sufficient to image single DNA molecules. Briefly, the donor molecules are specifically excited by a laser of appropriate wavelength that is incident on a Pellin Broca prism at the critical angle necessary to achieve TIRF (Figure 3A).²⁶ Polymerase molecules labeled with an acceptor fluorophore are then introduced into the imaging chamber to bind to the immobilized DNA. Upon polymerase binding to DNA, if the donor and acceptor fluorophores are sufficiently near to one another, energy transfer (i.e., FRET) from the donor to the acceptor can occur (Figure 3A). The fluorescence emission of the donor is then spectrally separated from the acceptor by the utility of dichroic mirrors to form distinct donor and acceptor emission channels (Figure 3B). Subsequently, both donor and acceptor emissions are incident on an electron multiplying charged coupled device (EMCCD) camera to record the fluorescence intensities over time (Figure 3C). The FRET efficiency (E) is then calculated as $E = I_A / (I_A + I_D)$, where I_D and I_A are the fluorescence intensities of the donor and acceptor, respectively. Accordingly, polymerase binding events and subsequent protein conformational changes are reflected as anticorrelated decreases in the donor emission intensity and increases in the acceptor emission intensity. Notably, smFRET has been applied to study various biochemical systems, including DNA replication.^{29,59–67} However, of the many existing DNA polymerase smFRET studies, only a few have explored TLS.^{29,65,67}

A recent publication from our laboratory utilized smFRET to study the lesion bypass capabilities of Dpo4.²⁹ Dpo4, the lone Y-family DNA polymerase in *Sulfolobus solfataricus*, has been extensively studied due to its structural and functional similarities to the human Y-family polymerases.^{14,28} We sought to explore the effects of a templating 8-oxoG lesion on the dynamics of Dpo4 during DNA binding as well as nucleotide binding. By examination of single-molecule FRET trajectories of Dpo4 binding to immobilized DNA substrates with a dG base at the templating position, we first observed the dynamic behavior of the polymerase on undamaged DNA. Interestingly, Dpo4 was shown to interconvert between three primary, nonzero FRET efficiency states (Figure 4A). In any smFRET analysis, assigning physical significance to observed FRET states is challenging. For our study, we had to carefully analyze all control and experimental data as well as apply our knowledge of Dpo4 from previous stopped-flow FRET investigations^{41,42,68,69} before developing the proposed model. We assigned the mid-FRET efficiency state (FRET $\approx$ 0.6) to the polymerase bound to the DNA with the primer/template junction base pair occupying the position of nascent base pair in the active site (preinsertion state), whereas in the low-FRET efficiency state (FRET $\approx$ 0.4), Dpo4 had translocated along the DNA substrate by one base pair to permit the binding of an incoming nucleotide (insertion state). Beyond the location of the polymerase at the primer/template terminus, the low- and mid-FRET states are also associated with distinct conformations of the polymerase as suggested by our dwell time analysis of the low- and mid-FRET states, wherein the duration of time the polymerase remains at a particular FRET efficiency value before transitioning to another is quantified.

We found that the rate of interconversion ($2.8\text{--}4\text{ s}^{-1}$) between the low- and mid-FRET states was far too slow to be reporting on the actual DNA translocation event itself, as mistakenly suggested by another single-molecule research group,⁶⁶ which has been estimated to occur at rates of $>100\text{ s}^{-1}$.^{41,68,70–72} The rate of interconversion actually reports on domain motions that occur concomitant with translocation along the DNA substrate, as observed through the Cy5-labeled Finger domain in our smFRET study.²⁹ Accordingly, our FRET analysis was also reporting on the dynamics of the Finger domain during DNA binding as well as DNA translocation, which is consistent with the dynamic behavior of the Finger domain as well as other domains of Dpo4 previously elucidated by our research group through stopped-flow FRET studies.^{41,42,68,69,73} Interestingly, our experiments also detected the existence of a high-FRET state ($\text{FRET} \approx 0.8$), which we expect to be a unique binding mode of Dpo4 involving the Little Finger domain, although more work is necessary to better characterize this unique finding.

When Dpo4 was bound to DNA containing the 8-oxoG lesion at the templating position (Figure 4B), the results were largely unchanged compared to those with the undamaged DNA substrate (Figure 4A). However, we did detect subtle differences between the experiments using 8-oxoG-containing DNA compared to those with undamaged DNA, including a shift in the number of FRET events at the low-FRET state (50% with the 8-oxoG-containing DNA (Figure 4D) versus 35% with the undamaged DNA (Figure 4C)) as well as increased polymerase binding times and slower dissociation kinetics from the lesion-containing DNA substrate. Therefore, more polymerase molecules were observed to be in the productive insertion state ready to accept an incoming nucleotide as well as spent longer periods of time on the 8-oxoG-containing DNA substrate without dissociating. Accordingly, we suggested that the 8-oxoG lesion modestly stabilized the formation of productive binary complexes. This assertion was further supported by experiments wherein the correct nucleotide, dCTP, was included in the imaging chamber in the presence of noncatalytic Ca^{2+} to form ternary complexes ($\text{E''} \cdot \text{DNA} \cdot \text{dCTP}$, Scheme 1) of either Dpo4 with undamaged or 8-oxoG-containing DNA. Interestingly, the presence of the correct nucleotide completely shifted all FRET events to the low-FRET state and significantly enhanced the FRET event durations. In fact, Dpo4 remained bound to DNA 5–10-fold longer in the presence of a correct over an incorrect dNTP. Furthermore, we observed that Dpo4, in the presence of dCTP, remained bound to 8-oxoG-containing DNA nearly 2-fold longer than undamaged DNA. In this way, we were able to observe the nucleotide selectivity of a Y-family DNA polymerase on undamaged as well as damage-containing DNA and preference for the 8-oxoG lesion at the templating position. Notably, Dpo4 has been shown to replicate more efficiently across from 8-oxoG than from normal dG,³⁹ and our data suggest that this occurs as a result of stabilization of the ternary complex of the polymerase. Consistently, these findings are in agreement with previous structural studies that have established that Arg332 in the Dpo4 active site forms specific interactions with 8-oxoG.⁷⁴ Our single-molecule investigation of the binding modes and kinetics of Dpo4 is largely consistent with a previous smFRET study of Dpo4 by the Rueda and Romano research laboratories wherein Dpo4 binds to undamaged DNA in at least two FRET states with the low-FRET state stabilized by correct nucleotide binding.⁶⁶ Another smFRET study by these groups investigated the effects of the bulky DNA adducts 2-aminofluorene and *N*-acetyl-2-aminofluorene on Dpo4

binding conformations and activity when the lesions were located at the templating or junction base pair positions.⁶⁷ Their results suggest that these bulky lesions significantly distort the structure of the binary complex as compared to results with unmodified DNA or our results with the nonhelix distorting lesion 8-oxoG. Consistent with our results with 8-oxoG, the addition of the correct nucleotide again induced a stable ternary complex (E''·DNA·dCTP, Scheme 1) despite the DNA damage in the template strand. Collectively, their study and ours illuminate key details of the response of Dpo4 to two bulky DNA adducts and a small, major oxidative lesion, respectively, and help to explain their mechanisms of mutagenicity. It is clear from these studies that Dpo4 is better suited to faithfully replicate opposite 8-oxoG, whereas the bulkier amine adducts more significantly affect Dpo4 binary and ternary complexes to contribute to misincorporation events.

For future investigation, it will be interesting to see how the extension step of TLS by Dpo4, which has been shown to be more significantly disrupted by DNA damage,^{75,76} is affected by the 8-oxoG lesion at the single-molecule level as well as how the inclusion of bulkier, DNA-helix distorting lesions, such as cyclobutane pyrimidine dimers or nitropolyaromatic hydrocarbon-generated lesions, affect the single-molecule behavior of Dpo4. Furthermore, it remains to be seen if other Y-family polymerases act similar to Dpo4 when they bypass a lesion and then subsequently extend the lesion bypass product. Moreover, we are excited at the prospect of utilizing single-molecule spectroscopy to investigate the complexities of polymerase switching during TLS. When a replicative polymerase is stalled by a DNA lesion during DNA replication, it is replaced by a Y-family polymerase, which is expected to bypass the damage in a process known as polymerase switching.^{77–80} This event is thought to be mediated by the ring-like clamp protein proliferating cell nuclear antigen (PCNA), which may simultaneously bind to both the replicative and Y-family DNA polymerases.^{81–83} Although polymerase switching is a vital, damage tolerance response for the cell, the details of the process are not well-defined and several competing mechanisms exist to describe its functioning.^{77–80} Application of smFRET to study polymerase switching will undoubtedly yield key mechanistic insight and clarity into the protein–protein and protein–DNA dynamics at the stalled replication fork.

CONCLUSIONS

Taken together, our detailed analyses of DNA damage tolerance and repair by cutting-edge time-dependent crystallographic and single-molecule methodologies have significantly increased our understanding of these vital life processes. In the coming years, it will be interesting to see how these techniques will continue to inform on the mechanisms of BER enzymes as well as the putative coordination and interactions between them. Moreover, we are excited about the prospect of further clarifying the underlying mechanisms of mutagenic translesion DNA synthesis by X- and Y-family DNA polymerases by these methods. In all, the technological advances in enzymology described in this review have informed ground-breaking research to yield paradigm-shifting results, and we expect that future applications will be equally impactful to the field.

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Biography

Austin T. Raper graduated *Summa Cum Laude* from the University of Mount Union in 2013 with a B.S. in Biochemistry. As a fellow of the Chemistry and Biology Interface Training Program and member of the Ohio State Biochemistry Program, he is currently conducting research in the laboratory of Dr. Zucai Suo (Department of Chemistry and Biochemistry) at The Ohio State University to earn a Ph.D. in Biochemistry. Austin's research focuses on characterizing enzymes critical for DNA lesion bypass and damage repair using biophysical and presteady-state kinetic methodologies.

Andrew J. Reed received his B.S. in Biochemistry from the University of Mount Union in 2013. He is currently a member of the Ohio State Biochemistry Program and is conducting research in the laboratory of Dr. Zucai Suo (Department of Chemistry and Biochemistry) at The Ohio State University in pursuit of a Ph.D. in Biochemistry. Andrew is investigating enzymes critical for DNA base excision repair and lesion bypass through X-ray crystallographic and presteady-state kinetic techniques.

Varun V. Gadkari received his B.S. in Biochemistry from The Ohio State University in 2012. Currently, he is pursuing a Ph.D. in Biochemistry in the laboratory of Dr. Zucai Suo (Department of Chemistry and Biochemistry) at The Ohio State University and is a member of the Ohio State Biochemistry Program. Varun is working to understand DNA damage tolerance using biophysical and presteady-state kinetic approaches.

Zucai Suo received both B.S. and M.S. in chemistry from Fudan University in China and his Ph.D. in chemistry from Pennsylvania State University in 1997. He was then a Jane Coffin Childs Memorial Fund Postdoctoral Fellow for two years at Harvard Medical School. Subsequently, he worked as a senior biochemist at Eli Lilly for 15 months. Since 2001, Dr. Suo has been a professor at The Ohio State University. His research interests include kinetic, dynamic, and structure–function relationship studies of DNA polymerases and antiviral and anticancer drug discovery. He is a fellow of the American Association for the Advancement of Science.

ABBREVIATIONS

8-oxoG	8-oxo-7,8-dihydro-2'-deoxyguanine
hPolβ	human DNA polymerase beta
smFRET	single-molecule Förster resonance energy transfer
FRET	Förster resonance energy transfer
BER	base excision repair

TLS	translesion synthesis
TIRF	total internal reflection fluorescence
EMCCD	electron multiplying charged coupled device

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Figure 1.

Time-dependent X-ray crystallography. (A) Cartoon representation of time-dependent X-ray crystallography method. Crystal trays are set up in the necessary conditions to grow protein crystals of precatalytic ternary polymerase complexes in the presence of Ca^{2+} . The isolated crystals are transferred to a cryosolution that contains magnesium (Mg^{2+}) to initiate the reaction in crystallo by exchanging the bound Ca^{2+} with Mg^{2+} . The crystals are soaked in the Mg^{2+} cryosolution for varying times ($t_1, t_2, t_3, \dots$) and then transferred to liquid N_2 to freeze-quench the reaction before X-ray diffraction experiments. (B–D) Structural progression of single-nucleotide incorporation catalyzed by hPolβ (PDB ID: 4RPX, 4RPZ, and 4RQ0).²⁵ (B) Precatalytic ternary complex of hPolβ with correct incoming dCTP paired with templating 8-oxoG with bound A- and B-site divalent metal ions (green spheres, M_A and M_B , respectively). (C) Reaction state structure of hPolβ during phosphoryl transfer with the incoming nucleotide modeled partially as dCTP and dCMP to match the increasing electron density (not shown) between the primer 3'-OH and the α -phosphate of dCTP and the decreasing electron density (not shown) between the α - and β -phosphates of dCTP. The third divalent metal ion occupies the C-site (M_C) and is coordinated by the nonbridging oxygen atoms of the α - and β -phosphates and water molecules (not shown). (D) Postcatalytic complex of hPolβ following complete incorporation of dCTP opposite 8-oxoG with M_C and pyrophosphate (PP_i) still bound. Notably, M_A is modeled as Na^+ (purple sphere). (B–D) Each panel shows a cartoon model of the N-Helix of the thumb domain in the closed conformation.

Figure 2.

Active site views of hPol β with templating 8-oxoG or incoming 8-oxo-dGTP. (A) Templating *anti*-8-oxoG with incoming dCTP (PDB ID: 4RPX).²⁵ (B) Templating *syn*-8-oxoG with incoming dATP (PDB ID: 4RQ3).²⁵ (C) Incoming *anti*-8-oxo-dGTP with templating dC (PDB ID: 4UBC).²⁴ (D) Incoming *syn*-8-oxo-dGTP with templating dA (PDB ID: 4UAW).²⁴

Figure 3.

Single-molecule FRET. (A) Modified, donor-labeled DNA is immobilized via the biotin–NeutrAvidin interaction. Donor molecules (green spheres) are specifically excited by a laser of appropriate wavelength using total internal reflection fluorescence (TIRF). Acceptor-labeled polymerase (red spheres) binding to DNA results in energy transfer (i.e., FRET). (B) The resulting fluorescence emissions of the donor and acceptor are spectrally separated to form separate donor and acceptor emission channels. (C) Movies of single molecules are recorded, and colocalized fluorescence intensities in the donor and acceptor emission channels are extracted by customized software. Anticorrelated changes in the donor and acceptor fluorescence intensities, shown in green and red, respectively, indicate FRET events.

Figure 4. Single-molecule analysis of Dpo4 binding to undamaged or 8-oxoG-containing DNA.²⁹ (A,B) The top panels show representative single-molecule trajectories of Dpo4 binding to undamaged DNA (A) or 8-oxoG-containing DNA (B) with donor and acceptor fluorescence intensities shown in green and red, respectively. The bottom panels display the corresponding, calculated FRET efficiency in blue and the idealized FRET efficiency from a Hidden Markov analysis in black. Three nonzero FRET states are clearly evident in each trajectory: high-, mid-, and low-FRET. FRET efficiency histograms of Dpo4 binding to undamaged (C) or 8-oxoG-containing DNA (D) with individual Gaussian peak fits for each FRET state are shown as overlapping black dashed lines, and the Gaussian sum is shown as

the solid black line. The respective population percentages for each FRET state are indicated in black.

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Scheme 1. Proposed Mechanism of Nucleotide Incorporation Catalyzed by a DNA Polymerase^a

^aE, E', and E'' represent three different conformations of the DNA polymerase sampled during correct nucleotide incorporation. E* represents the non-productive, pre-translocated binary complex.