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Active site interactions impact phosphoryl transfer during replication of damaged and undamaged DNA by *Escherichia coli* DNA Polymerase I

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Abstract

Replicative DNA polymerases are able to discriminate between very similar substrates with high accuracy. One mechanism by which *E coli* DNA polymerase I checks for Watson-Crick geometry is through a hydrogen bonding fork between Arg668 and the incoming dNTP and the minor groove of the primer terminus. The importance of the Arg-fork was examined by disrupting it with either a guanine to 3-deazaguanine substitution at the primer terminus or the use of a carbocyclic deoxyribose analog of dUTP. Using thio-substituted dNTPs and differential quench techniques, we determined that when the Arg-fork was disrupted, the rate limiting step changed from a conformational change to phosphodiester bond formation. This result indicates that Arg668 is involved in the phosphoryl transfer step. We examined the role of the Arg-fork in the replication of four DNA damaged templates, *O*⁶-methylguanine (*O*⁶-mG), 8-oxo-7,8-dihydroguanine (oxoG), *O*²-[4-(3-pyridyl)-4-oxobutyl]thymine (*O*²-POB-T), and *N*²-[(7*S*,8*R*,9*S*,10*R*)-7,8,9,10-tetrahydro-8,9,10-trihydroxybenzo[*a*]pyren-7-yl]-guanine (*N*²-BP-G). In general, the guanine to 3-deazaguanine substitution caused a decrease in k_{pol} that was proportional to k_{pol} over 5-orders of magnitude. The linear relationship indicates that the Arg668-fork helps catalyze phosphoryl transfer by the same mechanism with all the substrates. Exceptions to the linear relationship were the incorporations of dTTP opposite G, oxoG, and *O*⁶mG, which showed large decreases in k_{pol} , similar to that exhibited by the Watson-Crick base pairs. It was proposed that the incorporation of dTTP opposite G, oxoG, and *O*⁶mG occurred via Watson-Crick-like structures.

Graphical abstract

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Supporting Information Available. Figure S1 to S15 show time courses and rate constant versus dNTP concentration plots of the various oligodeoxynucleotides. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

DNA polymerases are essential to the high fidelity replication of the genome. High fidelity polymerases, without the proofreading activity, are able to insert the correct dNTP 10^4 to 10^6 - fold more often than the wrong dNTP.¹ While the basis for accurate replication was noted by Watson and Crick,² the mechanism underlying fidelity is not fully understood.³ The high fidelity is remarkable because the DNA polymerases (pols) (1) must recognize four different base-pairs as the correct substrates, (2) the position of the nascent base pair, where the selectivity is determined, is $10 \text{ \AA}$ from the site of chemistry, and (3) the flexibility of the DNA attenuates differences in base pair geometry from affecting structure of the phosphate backbone.^{4–6} The energetics of base pairing cannot solely account for the high fidelity of DNA replication, and thus the polymerase must play an active role in dNTP selectivity.^{7–9} The current paradigm is that nucleotide selectivity largely depends on a combination of thermodynamics and geometric selection for the shape and size of Watson-Crick base pairs.^{3, 8, 10–15}

While DNA polymerases have been categorized into multiple families based upon amino acid sequence, they all share similar structural features and catalyze the same reaction. Since the discovery of DNA polymerase I in *Escherichia coli*, A-family polymerases have served as a prototype for understanding the mechanisms for catalysis and fidelity during DNA replication. The overall structure of a polymerase has been referred to as a right hand, containing palm, fingers and thumb subdomains.¹⁶ The kinetic scheme for A-family polymerases has been extensively examined and found to contain multiple intermediates as shown in Scheme 1.^{17–22} The DNA initially binds to the polymerase in an open configuration (ED_n), allowing dNTPs to bind to the catalytic site (step 1). The fingers close down over the DNA and dNTP to form the catalytically active closed conformation (step 2). Crystallography experiments show a large conformational change in which the fingers subdomain closes down over the active site.^{23–26} However, this movement is rapid, and occurs prior to smaller motions in the active site, perhaps due to the binding of the catalytic Mg^{2+} .^{27–29} Phosphoryl transfer then occurs (step 3), followed by opening of the fingers (step 4) and loss of pyrophosphate (step 5).

Polymerase selectivity can be viewed as multiple checkpoints along the reaction pathway.³⁰ The first fidelity checkpoint is the binding of the dNTP to the polymerase/DNA complex. The affinity of the incorrect dNTP to the polymerase-DNA complex is lower than for the correct dNTP. The second checkpoint is in the formation of the closed complex. While the

rate of formation of the closed complex is similar for correct and incorrect base pairs, the energy of the closed complex with the wrong dNTP is higher, leading to an increase in the k_{-2} .^{15, 28, 29} These two checkpoints reject incorrect nucleotides before phosphoryl transfer occurs.

A third checkpoint is phosphoryl transfer. During correct base pair formation, phosphoryl transfer (k_3^r) is rapid and the rate limiting step is the preceding conformational change (k_2^r).^{17–22} The rate of phosphodiester bond formation decreases and becomes rate limiting during mispair formation.^{19–21}

Fluorescent studies indicate that the closed complex with the wrong dNTP (E^*D_nW) has a different conformation than that with the correct dNTP (E^*D_nR).^{15, 29, 31} Based upon the FRET signal, this complex was described as partially open.²⁹ Crystallographic studies have identified closed complexes with G/T and A/C mispairs that differ from those observed with correct base pairs.^{32, 33} In this complex, the mispair is in a wobble configuration and the protein adopts a configuration that was termed “ajar” to represent that the protein conformation is between an open and closed complex.³² The formation of a low reactivity complex with the incorrect dNTP may be an active process.^{15, 34, 35} When the correct dNTP binds the induced fit conformation change creates an active complex, but if a wrong dNTP binds then the induced fit conformation contains misaligned residues that inefficiently catalyze phosphoryl transfer and promote rapid release of the dNTP from the complex.^{3, 15} If this mechanism occurs then critical amino acid residues will have different positions and thus different roles during correct versus incorrect base pair formation. In this manuscript we probed the mechanisms by which Arg668 of *E. coli* DNA polymerase I functions to reduce phosphodiester bond formation during mispair formation. This residue is conserved in A-family polymerases (Klenow fragment of *E. coli* DNA polymerase I (KF) 668, T7 DNA polymerase (T7) pol, 429; large fragment of *Thermus aquaticus* DNA polymerase I (Klentaq), 573; large fragment of *Bacillus stearothermophilus* DNA polymerase I (BF), 615; pol ν , 571; pol γ , 853).³⁶ Crystal structures show that this residue makes a hydrogen-bonding fork between the minor groove of the primer terminus and the ring oxygen of the deoxyribose moiety of the incoming dNTP as shown in Figure 1A.^{23–25, 37} Interactions with the minor groove were proposed to be important for DNA replication fidelity because the O^2 -position of pyrimidines and the $N3$ -position of purines occupy similar spatial orientations and chemical properties (hydrogen bond acceptors) irrespective of the base pair.³⁸ Thus, the minor groove can be used to check for Watson-Crick geometry. Site-directed mutagenesis studies have supported the important role of Arg668 in catalysis and fidelity.^{39–41}

We previously utilized 3-deazaguanine and 1-[(1*R*, 3*S*, 4*R*)-3-hydroxy-4-(hydroxymethyl)cyclopentyl]-2,4(1*H*, 3*H*)-pyrimidinedione (dcUTP) to evaluate the role that the KF-Arg668-DNA hydrogen bonding fork plays in catalysis and fidelity.^{42, 43} As shown in Figure 2, these compounds are nucleotide analogs in which a nitrogen is replaced by carbon (3DG) and an oxygen by carbon (dcUTP). While these substitutions have minimal steric impact, they disrupt hydrogen bonding interactions. We found that either substitution produced a 1000-fold decrease in k_{pol} but that the combination of the substitutions did not

additionally decrease reactivity.⁴² Therefore, breakage of one hydrogen bond eliminated the effect of the Arg-fork.

We further found that the Arg-fork played an important role in catalyzing correct base pair formation but did not help catalyze mispair formation.⁴³ This result is consistent with fluorescence and crystallographic studies that indicate the polymerase adopts different conformations when bound to the right versus wrong dNTP.^{15, 32} However, our conclusions were based upon two types of substrates, correct base pairs and mismatches, which react either very rapidly or slowly. Thus, our bimodal results may have been due to the use of good and poor substrates. To more fully examine the possibility that KF exists in two distinct catalytic states we examined the role that Arg668 plays in replication of a set of the damaged DNA templates, *O*⁶-methylguanine (*O*⁶-mG), 8-oxo-7,8-dihydroguanine (oxoG), *O*²-[4-(3-pyridyl)-4-oxobutyl]thymine (*O*²-POB-T), and *N*²-[(7*S*,8*R*,9*S*,10*R*)-7,8,9,10-tetrahydro-8,9,10-trihydroxybenzo[*a*]pyren-7-yl]-guanine (*N*²-BP-G). (See Figure 2 for structures). *O*⁶mG and oxoG react with KF with rates intermediate between mismatch and correct base pair formation, while dNTPs are incorporated opposite *O*²-POB-dG and *N*²-BP-dG at rates much less than mismatch formation. We found that breaking the Arg-fork decreased the rate of reaction by reducing the chemical step of phosphoryl transfer. We also found that the effect of breaking the Arg-fork was proportional to the rate of reaction for most mismatches and damaged templates. Three exceptions to this observation were the incorporations of dTTP opposite G, *O*⁶mG and oxoG. With these substrates, disruption of the Arg-fork produced large decreases in rate, similar to those observed in correct base pair formation. The dual nature of the effect suggests that the role that Arg668 plays in replication differs with respect to the geometry of the newly forming base pair.

Experimental procedures

General

[³²P]ATP was purchased from Perkin Elmer at 6000 Ci/mmol. T₄ polynucleotide kinase and the Klenow fragment of *E. coli* DNA polymerase I with the exonuclease activity inactivated (KF⁻) were obtained from US Biological (Salem, MA). The dNTPs (ultrapure grade) were purchased from Amersham Biosciences, 5'-(α -P-thio)triphosphates were purchased from TriLink Biotechnologies (San Diego, CA), and the concentrations were determined by UV absorbance.⁴⁴ 1-[(1*R*, 3*S*,4*R*)-3-hydroxy-4-(hydroxymethyl)cyclopentyl]-2,4(1*H*,3*H*)-pyrimidinedione (dcUTP) was synthesized as described.⁴² The phosphoramidites for *O*⁶mG, and oxoG were purchased from Glen Research (Sterling, VA). The oligodeoxynucleotides containing 6-amino-1-(2-deoxy- β -D-*erythro*-pentofuranosyl)-1,5-dihydro-4*H*-imidazo[4,5-*c*]pyridine-4-one (3DG), *O*²-POB-T, and *N*²-BP-dG were synthesized as described.⁴⁵⁻⁴⁷ The sequence of the template strand was 5'-CTG CGA CAX CTG CGT CTG CGG TGC-3' with X being A,C,G,T, *O*⁶mG, oxoG, *N*²-BP-G, and *O*²-POB-T. The primer strand was 5'-GCA CCG CAG ACX-3' with X being G or 3DG. The primer strands were ³²P-labeled with γ -[³²P]ATP in a reaction catalyzed by T₄-polynucleotide kinase, the oligomer was separated from low molecular weight impurities with a spin column containing Sephadex G-25 and the primers were annealed with a 25% excess of the template strands.⁴⁸

Polymerase kinetics

Rapid reactions were conducted with a RQF-3 rapid quench instrument (KinTek Corp, Austin TX) while slower reactions were performed manually. The reactions were initiated by the addition of equal volumes of dNTP in 10 mM MgCl₂ to DNA-KF in 100 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 5 mM DTT, 200 µg/mL BSA at 25 °C. Typically the DNA concentration was 25 nM and the polymerase concentration was 100 nM. The concentration of dNTPs varied from 0 to 2000 µM. Manually conducted reactions were quenched with an equal volume of STOP solution (10% 300 mM EDTA (pH 8.0), 90% formamide, 25 mg/mL bromophenol blue, and 25 mg/mL xylene cyanol). The rapid reactions were quenched by the addition of 300 mM EDTA at various times. STOP solution (100 µL) was added in order to load the samples onto the polyacrylamide gel. An alternative quench reagent was 1.8 N HCl. The resulting acidic sample mixtures were extracted with 1 ml CHCl₃/*iso*-amyl alcohol (24:1) then neutralized with NaOH.

During the pulse-chase experiments, the reactions were initiated by the addition of 2.5 µM [α -³²P]dCTP 10 mM MgCl₂ to 25 nM unlabeled DNA and 100 nM KF. The reactions were quenched with 1mM dCTP for 30 s followed by 1 mL 1 N HCl.

Product analysis by PAGE

The progress of the reaction was analyzed by denaturing PAGE in 20% acrylamide (19:1, acrylamide-*N,N'*-methylene bisacrylamide), 7 M urea in 1X TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M Na₂EDTA). The size of the gel was 40 × 33 × 0.04 cm and was run at 2000 V for 2 - 2.5 h. The radioactivity on the gel was visualized with Typhoon 9200 (GE Healthcare). The progress of the reaction was quantitated by dividing the total radioactivity in the product band(s) by the radioactivity in the product and reactant bands. During the pulse-chase experiments, ³²P-labeled oligodeoxynucleotide was separated from unreacted [α -³²P] dCTP by PAGE. The relative extent of reaction was quantitated by the total radioactivity in the product band.

Data analysis

Data were fitted by nonlinear regression using the program Prism version 5 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Data from the reactions were fitted to equation 1, where P is the product formed, A is the total amount of DNA reacted, and k is the first order rate constant for the dNTP incorporation. If the reactions exhibited two phases then the data were fit to equation 2 in which A is the burst amplitude and k_{ss} is the rate constant of the slow phase. The k values for these experiments were fitted to equation 3, where k_{pol} is the maximum rate of dNTP incorporation and K_d is the apparent equilibrium dissociation constant for the interaction of dNTP with the polymerase-DNA complex.

$$P=A(1-e^{-kt}) \quad (1)$$

$$P = A(1 - e^{-kt}) + k_{ss}t \quad (2)$$

$$k = k_{pol} [dNTP] / ([dNTP] + K_d) \quad (3)$$

Results

We previously determined that the KF-Arg668-DNA hydrogen bonding fork played a critical role in in catalysis and fidelity.^{42, 43} We concluded that the Arg-fork played two roles in catalysis, in that it was very important in correct base pair formation but not important in mispair formation. Our conclusions were based upon two types of substrates, correct base pairs and mismatches, which react either very rapidly or slowly. Thus, our bimodal results may have been due to use of good and poor substrates. In this manuscript we further tested this hypothesis by examining an array of DNA substrates with a broader reactivity range using four mutagenic bases *O*⁶mG, oxoG, *O*²-POB-T and *N*²-BP-G (Figure 2). The rates of incorporation of dNTPs opposite *O*⁶mG, oxoG are intermediate between correct base pairs and mismatches. We also examined the effect with *O*²-POB-T and *N*²-BP-G in the template, because KF catalyzes the insertion of dNTPs opposite these nucleotides at rates slower than mismatch formation.

Arg668-fork is crucial to phosphoryl transfer step

First, we examined the kinetic step in which the Arg-fork plays its crucial role, by determining which step in the reaction is rate-limiting when the Arg-fork is broken. We employed two modified substrates, 3DG at the primer terminus and dcUTP as the incoming dNTP (Figure 2). With each substrate, a heteroatom that forms a hydrogen bond with Arg668 is replaced by a carbon, thereby eliminating one prong of the hydrogen-bonding fork, as shown in Figure 1. Previously, we showed that these substrates reduced the rate of correct base pair formation by three-orders of magnitude, and that employing both substrates simultaneously did not additionally decrease the rate of reaction.⁴² Two complementary methods were employed to determine the rate limiting step with these two substrates: the thio-effect and alternative quench experiments.

dNTPs in which the α -phosphate is modified with sulfur have been employed as probes for the rate limiting step in DNA polymerase reactions.^{21, 40, 49} It has been shown with KF that the thio-substitution reduces the rate of phosphodiester bond formation, but not the preceding conformational change.³⁹ Thus, reduction of the rate of reaction with dNTP α S substitution is consistent with a rate limiting phosphoryl transfer step. However, if dNTP α S substitution does not reduce the rate, then the rate limiting step would be a conformational change.

In Figure 3, we utilized dCTP α S as a probe for the rate limiting step with G (panel A) and 3DG (panel B) at the primer terminus. As shown in Figure 3A, the rate of dCTP α S incorporation was equal to that of dCTP, indicating the rate limiting step is not

phosphodiester bond formation. In contrast, with 3DG at the primer terminus, thio-substitution significantly decreased the rate of the reaction (Figure 3B). This result indicates that when the arginine fork is broken with 3DG at the primer terminus the rate limiting step is phosphodiester bond formation.

Figure 3C and D show the pulse-quench experiment. These reactions were initiated by the addition of [α - 32 P] dCTP and MgCl_2 to unlabeled DNA and KF. The reactions were quenched by the addition of an excess of unlabeled dCTP or HCl. While the HCl will stop the reaction immediately, dCTP will allow internal equilibrations and reactions to proceed until either product or dCTP diffuses from the active site. If the rate limiting step is the conformational change, then during the dCTP quench, the [dNTP-DNA-polymerase] closed conformation complex will react to form more product than that observed in the HCl quench. However if phosphodiester bond formation is rate limiting, the ternary complex will not react, and the dCTP and HCl quenched reactions will have equal amounts of product.^{50, 51} The excess product formed in the dCTP quench reaction in Figure 3C is indicative of a rate limiting conformational change preceding phosphodiester bond formation with G at the primer terminus. In contrast, the results in Figure 3D, with 3DG at the primer terminus, is consistent with rate limiting phosphoryl transfer.

While we utilized the pulse-chase versus HCl quench to examine the rate limiting step during the incorporation of dCTP with 3DG at the primer terminus, we cannot use the same techniques to probe the rate limiting step with dcUTP because we lacked the α - 32 P-labeled triphosphate. In an alternative experiment, we examined the progress of the reaction under two quench conditions, EDTA and HCl. HCl will stop the reaction immediately, but EDTA will allow internal equilibrations and reactions to proceed until Mg^{2+} diffuses from the active site. It was previously found with KF-catalyzed single nucleotide incorporation reactions that while the rate constants produced by EDTA and HCl quenched reactions are identical, the EDTA quenched reaction produced a larger burst amplitude.^{50, 51} Thus, EDTA quenches the reaction in a similar manner to the pulse-chase in which internal equilibrations are allowed to proceed until the active site opens to release the contents. The EDTA quench has an advantage in that the [α - 32 P]dNTP is not needed to detect the reaction.

In Figure 3E and F we compared the effect of the EDTA vs HCl quench on the G to 3DG substitution at the primer terminus. The results were identical to that observed in the pulse-chase experiments. The excess product formed in the EDTA quench reaction in Figure 3E is indicative of a rate limiting conformational change preceding phosphodiester bond formation with G at the primer terminus. In contrast, the results in Figure 3F, with 3DG at the primer terminus, is consistent with rate limiting phosphoryl transfer.

The EDTA vs HCl quench conditions were then utilized to examine the rate limiting step for the incorporation of dcUTP opposite dA. In Figure 3G, the excess product produced by the EDTA quench is consistent with a rate limiting conformational change prior to a rapid phosphoryl transfer step during the incorporation of dUTP opposite dA. In Figure 3H, the equal amounts of product produced by both quench methods indicates that the rate limiting step is phosphoryl transfer.

In summary, the arginine fork is important in the phosphoryl transfer step. When the fork is disrupted, by either the G to 3DG substitution at the primer terminus or the dUTP to dcUTP substitution, the rate of phosphoryl transfer decreases to become rate limiting.

Effect of breaking Arg-fork with undamaged DNA

We analyzed the effect of breaking the Arg-fork with the G to 3DG substitution at the primer terminus as previously described.⁴³ The time course was fit to a first-order or burst equation (Equation 1 or 2). The rate constant, k , was obtained at different dNTP concentrations. The parameters k_{pol} and K_d were obtained by fitting k to $[\text{dNTP}]$ according to equation 3. The data for G as the template were completely reanalyzed. The data for the other undamaged templates were checked for consistency with previous experiments. The parameters are presented in Table S1. The k_{pol} for correct base pair formation decreased an average of 500-fold when the Arg-fork was broken with the 3DG substitution at the primer terminus. During mispair formation, save for one exception, the effect on k_{pol} was small, and varied from a 30-fold decrease to a 2-fold increase. The exception was the insertion of dTTP opposite G, in which the k_{pol} decreased 700-fold, similar to that observed during correct base pair formation.

The G to 3DG substitution reduced the affinity of the dNTP to a lesser extent. The affinity of the correct dNTP decreased by 3 to 14-fold, while the affinity of the incorrect dNTPs were generally unaffected, exhibiting a 2-fold increase to 2-fold decrease in K_d .

In summary, the data is consistent with a mechanism in which the Arg-fork helps to catalyze correct base pair formation but not mispair formation. The selection occurs in both binding and phosphoryl transfer. The presence of nitrogen versus carbon at the 3-position of guanine at the primer terminus enhanced the affinity of the correct dNTP up to 10-fold. A greater effect is observed in the chemistry step in which the N3 enhances the k_{pol} value up to 1400-fold. As a result, we infer that the Arg-fork is a positive selection factor for correct dNTP incorporation but does not actively select against mispair formation. The exception, noted above, was the insertion of dTTP opposite G, in which the k_{pol} decreased 700-fold decrease. The magnitude of this decrease in k_{pol} is similar to that observed during correct base pair formation.

To visualize this data, the effect of the G to 3DG substitution at the primer terminus is presented in Figure 4. The x-axis is the $\log(k_{\text{pol}}^{\text{G}})$ and the y-axis is the $\log(k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{\text{3DG}})$. Since the data is plotted on a log scale, the x-axis is proportional to the transition state energy ($\Delta G^\ddagger$) of the reaction, while the y-axis is the change in transition state energy ($\Delta \Delta G^\ddagger$) caused by the 3DG substitution. The undamaged templates are represented by the filled symbols, while the modified templates are represented by open symbols. The color represents the incoming dNTP. For example the right most data point, with the largest $k_{\text{pol}}^{\text{G}}$ is the insertion of dATP (black) opposite T (filled diamond). The left most data point represents the insertion of dTTP (blue) opposite N^2 -BP-G (open triangle). The data points on the lower left have $k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{\text{3DG}}$ values near 1, indicating that the 3DG substitution had little to no effect on the k_{pol} parameter.

Role of Arg-fork with $\mathcal{O}^6\text{mG}$

$\mathcal{O}^6\text{mG}$ is a very promutagenic base as dTTP is incorporated opposite it very readily for many polymerases.^{52–54} The facile insertion of dTTP opposite $\mathcal{O}^6\text{mG}$ has been proposed to be due to its ability to form a Watson-Crick-like structure in the active site of polymerases as illustrated in Figure 5A.^{55, 56} Structural studies have shown that the $\mathcal{O}^6\text{mG}$ base pair with dC or dT can exist in multiple configurations, some of which are illustrated in Figure 5.^{54, 57–61} While atomic substitution supports the Watson-Crick-like structure during replication,⁶² crystallography experiments have identified the distorted Watson-Crick structure, illustrated in Figure 5B and C, bound to BF.⁶³ With both base pair combinations, Arg614 of BF, is in position to form the hydrogen bond fork between the dNTP and primer terminus. Kinetic analysis of KF-catalyzed bypass of $\mathcal{O}^6\text{mG}$ was consistent with a kinetic scheme in which the rate limiting step is phosphodiester bond formation for both dCTP and dTTP incorporation.⁶⁴

The [dNTP]-dependence on the k_{pol} with $\mathcal{O}^6\text{mG}$ as template is presented in Figures S2–S5. With G at the primer terminus, the k_{pol} and K_d for incorporation of dCTP and dTTP were approximately equal. The reduced base selectivity was due both to a decreased k_{pol} for dCTP and an increase in k_{pol} for dTTP. For dCTP, the k_{pol} was reduced 30-fold and the K_d was increased 4-fold relative to the incorporation of dCTP opposite G. The increased reactivity of dTTP opposite $\mathcal{O}^6\text{mG}$ was due to a 260-fold increase in k_{pol} and a 2-fold decrease in K_d . These values indicate that although $\mathcal{O}^6\text{mG}$ is a less reactive template than guanine, dCTP and dTTP can be incorporated opposite it more efficiently than mispair formation. The incorporations of dATP and dGTP opposite $\mathcal{O}^6\text{mG}$ were slow, with kinetic parameters similar to the incorporation opposite G. These results are consistent with results reported by others for A-family polymerases.^{52, 53, 63–65}

The G to 3DG substitution caused an 18 to 36-fold decrease in k_{pol} for the incorporation of dCTP, dATP, and dGTP opposite $\mathcal{O}^6\text{mG}$. This effect was smaller than the 400 to 1000 decrease in k_{pol} for correct base formation. From this result, it is inferred that the Arg668-fork stabilizes the transition state, but less than it does for correct base pair formation but more than most mispairs. The k_{pol} data points lie along the solid black line in Figure 4. The largest effect occurred in the incorporation of dTTP opposite $\mathcal{O}^6\text{mG}$, in which the 3DG substitution caused a 700-fold decrease in k_{pol} . The effect for dTTP is similar to that which has been observed for Watson-Crick base pairs. In particular, the effect of the 3DG substitution on dTTP incorporation is different than that for dCTP. This difference indicates that the role that Arg668 plays different roles in the incorporation dCTP and dTTP.

Role of Arg-fork with oxoG

oxoG is also very mutagenic, increasing the misincorporation of dATP. While dCTP is primarily inserted by KF, the ratio of dATP/dCTP is dependent on the polymerase.^{66–72} Ternary crystal structures between DNA and T7 pol have shown that dCTP pairs with oxoG in Watson-Crick geometry⁷³ and in a Hoogsteen base pair with dATP.⁷⁴ In both cases, the active site Arg is in position to make the Arg-fork with the incoming dNTP and the primer terminus. Pre-steady-state kinetic analysis of the KF – catalyzed incorporations of dCTP and

dATP opposite oxoG indicates that the rates of phosphodiester bond formation and the preceding conformational change are similar.⁶⁶

The [dNTP]-dependence on the k_{pol} with oxoG as template is presented in Figure S6 – S9. With G at the primer terminus, KF prefers the incorporation of dCTP over dATP, results similar to that previously observed.⁶⁶ We found the relative k_{pol}/K_d values for dCTP:dATP incorporation was 2.2. The approximately equal reactivity of dCTP and dATP was primarily due to a decreased reactivity of dCTP. The rate of incorporation of dCTP decreased due to a 57-fold drop in k_{pol} opposite oxoG relative to G. In contrast, the k_{pol} for the insertion of dATP opposite oxoG increased only 1.5-fold relative to the insertion of dATP opposite G. dGTP and dTTP were poorer substrates, with k_{pol} values at least 10-fold less than that for dATP.

The G to 3DG substitution caused a 120- and 68-fold decrease in the k_{pol} for the insertion of dCTP and dATP, respectively. The effect for dGTP was minimal, the k_{pol} decreased 1.4-fold. These effects are consistent with the trend along the black line in Figure 4, in that the presence of the nitrogen at the N3-position of guanine at the primer terminus is more important with the more reactive substrates. In contrast, the effect of the 3DG substitution on the incorporation of dTTP was very different. Despite the fact that dTTP was a much poorer substrate than dCTP or dATP, the G to 3DG substitution decreased the k_{pol} 1400-fold. The difference in the effect of the 3DG substitution indicates that the mechanism of incorporation of dTTP differs from that of dCTP.

Role of Arg-fork with N^2 -BP-G

N^2 -BP-G (Figure 2) is formed from metabolic activation of benzo[a]pyrene.⁷⁵ High fidelity polymerases bypass this adduct very slowly and is considered a blocking lesion. It is bypassed by Y-family polymerases in humans.⁷⁶ A crystal structure of N^2 -BP-dG paired with dC in the post insertion site of BF showed a Watson-Crick hydrogen bonding scheme between dC and N^2 -BP-dG.⁷⁷ However, the position of the BP-group in the minor groove distorted the positions of critical active site residues and consequently this structure was concluded to be unreactive. Molecular modeling experiments provides insight for the possible reactive conformations with T7 pol and BF.^{78–80} In these structures, the template N^2 -BP-G is in the *syn*-conformation, with BP-moiety pointing toward the major groove and the Hoogsteen hydrogen bonding face pointing toward the incoming dNTP. Our recent results support this hydrogen-bonding interaction.⁴⁷ In these models, the Arg is predicted to be in position to make the hydrogen-bonding interactions between the primer terminus and dNTP.⁸⁰

The dNTP concentration dependence on k_{pol} is shown in Figure S10–S13. dATP and dGTP are the best co-substrates with N^2 -BP-G, with k_{pol} values of 0.051 and 0.015 s⁻¹. These values are similar to the slowest k_{pol} s that we observed during mispair formation (See Table S1). The k_{pol} values for dCTP and dTTP incorporation are 10-fold slower. The 3DG substitution caused ~2-fold decrease in k_{pol} for the incorporation of dATP, dGTP, and dTTP, and a 5-fold increase for dCTP. These values fall near the solid line in Figure 4. Thus, we conclude that the Arg-fork plays a very small role in catalyzing these reactions.

Role of Arg-fork with O^2 -POB-T

O^2 -POB-dT is the most abundant bulky adduct derived from NNK and NNN, tobacco-specific nitrosamines.^{81, 82} In human cells, it is bypassed by pol η , ζ and REV1.^{48, 83} While dATP and dTTP are the best substrates, the k_{pol} values are less than that for mispair formation and are similar to the k_{pol} for incorporation of dCTP and dTTP opposite N^2 -BP-G (Table S1). The adduct is a very poor substrate for KF.⁸⁴ As shown in Figure S14–S15, the 3DG substitution did not affect the k_{pol} for the incorporation of dATP, and decreased the rate of dTTP incorporation 10-fold. The value for the dTTP incorporation falls below the black line in Figure 4. The data indicates that the nitrogen at the 3-position of guanine at the primer terminus does not play a role in the incorporation of dATP opposite O^2 -POB-dT, and a small role in the incorporation of dTTP.

Discussion

Arg668 is involved in phosphoryl transfer step

The mechanism of phosphodiester bond formation catalyzed by DNA polymerases is conserved among polymerases. The reaction involves the displacement of the pyrophosphate leaving group by the 3'-hydroxyl group of the primer terminus. The in-line placement of the nucleophile and leaving group^{23–26} and the stereochemistry of the α -phosphate of the dNTP⁸⁵ indicate a concerted S_N2 -type reaction. The precise positioning of the aspartate residues and Mg^{2+} ions lowers the pK_a of the 3'-hydroxyl of the primer, converting it into a better nucleophile,^{86–88} stabilizes the phosphorus pentavalent transition state, and increases the leaving group ability of the pyrophosphate. The optimum positioning of the amino acid residues and metal ions occurs during conformational changes that are rate limiting during correct base pair formation.

A-family polymerases contain a conserved active site Arg residue (KF 668, T7, 429; KlenTaq, 573; BF, 615; pol ν , 571; pol γ , 853).³⁶ This residue is unique in that it contacts both substrates, forming a hydrogen bonding fork bridging the primer terminus and the incoming dNTP in KlenTaq, BF, pol ν , and pol γ .^{23–26, 89} Disruption of this interaction in KF by amino acid substitution, or atomic substitution on the DNA or dNTP leads to decreased k_{cat} and k_{pol} values.^{39–43} When the Arg-fork is broken by the 3DG substitution at the primer terminus or the carbocyclic-dUTP, we found that the rate limiting step becomes phosphodiester bond formation (Figure 3). Two different mechanisms may account for these results. First, Arg668 may have a direct effect on the phosphodiester bond formation. Second, Arg668 may be involved in a checkpoint that determines if the polymerase adopts a high catalytic state.

Arg668 can have a direct effect on the chemistry via the approximation or proximity effect in which the enzyme increases the rate of reaction by positioning the substrates in reactive positions.⁹⁰ Although, Arg668 is 10 Å away from the site of bond formation, and thus does not directly participate in the chemical transformation, it does bridge the two substrates. Arg668 can accelerate phosphoryl transfer by positioning the two substrates. When the Arg-fork is broken by amino acid or atomic substitution, the reaction rate can decrease due to the

loss of the positioning of the Arg fork. During mispair formation or extension, the Arg-fork is misaligned, and would inefficiently help catalyze the reaction.

Alternatively, the fork may be involved in a pre-chemistry checkpoint. When both the terminal and newly forming base pairs are in the correct orientation, the Arg-fork can form and initiate conversion of the polymerase to a high catalytic state.¹⁵ When the Arg-fork cannot form, due to atomic substitution or due to the presence of mispairs, the conversion to the high catalytic state cannot occur. Thus, the amino acid residues would not be in the optimal position and phosphodiester bond formation would be rate limiting. The crystal structure of the dGTP/dT wobble base pair bound to BF supports this mechanism.³² In this structure, the Arg-fork is not formed and the polymerase is in an inactive ajar conformation.

Effect of 3DG substitution on replication of modified nucleotides

The effect of the 3DG substitution for all the base pair combinations is summarized in Figure 4. The slowest reacting substrates are the N^2 -BP-G and O^2 -POB-T damaged templates, on the left of the graph. The $k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{3\text{DG}}$ values for these substrates range from 0.2 to 10. Thus, based upon the G to 3DG substitution, we conclude that the Arg-fork plays only a small role in bypass of these adducts. As the k_{pol} value increases, the effect of the 3DG substitution also increases. The relationship between $k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{3\text{DG}}$ and $k_{\text{pol}}^{\text{G}}$ is linear from $k_{\text{pol}}^{\text{G}}$ values of 0.001 s^{-1} to 100 s^{-1} , five orders of magnitude.

Three base pairs are not on the black line, the incorporation of dTTP opposite oxoG, O^6 mG and G. The effect of the 3DG substitution was at least a 700-fold decrease in k_{pol} . Two properties that link these three base pairs is that the incoming triphosphate is dTTP and the template is guanine, or a modified guanine containing a small structural alteration, such as the O^6 -methyl or 8-oxo group. The large $k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{3\text{DG}}$ values for these substrates and the observation that these values are similar to those of the correct base pairs are consistent with a mechanism in which dTTP is inserted opposite these templates via Watson-Crick geometry.

Possible base pair structures between dTTP and G, O^6 mG, and oxoG are illustrated in Figure 5. Panels D-F show guanine as template, but these structures would also be appropriate for oxoG. G/T mispairs can potentially form via the wobble (Figure 5D),^{32, 91–93} a Watson-Crick-like structure with bifurcated hydrogen bonds (Figure 5E),^{94, 95} or the Watson-Crick structure with a minor tautomers (Figure 5F).⁹⁶ There has been chemical evidence that mispairs can form via ionized base pairs or minor tautomers.^{97, 98} Recently, crystal structures in the active site of polymerases have supported the role of Watson-Crick structures resulting from minor tautomers or ionized mispairs.^{33, 96} The high mutagenicity of O^6 mG was initially proposed to be via its ability to form a Watson-Crick base pair with dT (Figure 5A).^{55, 56} We previously reported results supporting Watson-Crick-like structure for the incorporation of dTTP opposite O^6 mG.⁶² In conflict with this conclusion, the base pair in the active site of BF was found to be the non-Watson-Crick structure in Figure 5B.⁶³ However, a crystal structure with pol β and Mn^{2+} showed a structure similar to the Watson-Crick structure in Figure 5A.⁹⁹

Thus, there is some evidence supporting Watson-Crick structure involving minor taster and ionized base pairs in mispair formation. While our data is consistent with Watson-Crick structures in the incorporation of dTTP opposite G and oxoG, it does not support them in the incorporation of dGTP opposite T or in the formation of AC mispairs.

Role of Arg668 in catalysis

We previously concluded that Arg668 had two roles in phosphodiester bond formation.⁴³ With correct base pairs, it was very important in catalyzing phosphoryl transfer, while with most mispairs, it was unimportant. This dichotomy of function was consistent with the proposal that correct and mispairs are formed via distinct mechanisms in which the polymerase existed in different conformations.^{3, 15}

The results presented in Figure 4 demonstrate that a linear relationship exists between $k_{\text{pol}}^{\text{G}}$ and $k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{3\text{DG}}$ for over five orders of magnitude. The linear relationship indicates that the Arg-fork catalyzes phosphoryl transfer by the same mechanism, with varying effectiveness over the range of substrates. The plot of $K_{\text{d}}^{\text{G}}/K_{\text{d}}^{3\text{DG}}$ vs $k_{\text{pol}}^{\text{G}}$ in Figure S16 shows a much smaller effect on K_{d} than on k_{pol} . If Arg668 existed in only two distinct conformations, then we would expect to observe a discontinuity in the results. Our data suggests that KF does not recognize good versus bad substrates in a precatalytic step that governs if the polymerase adopts a high or low catalytic conformation. In contrast, our data indicates that the mechanism of nucleotide selection is continuous over the array of substrates. The polymerase adopts multiple conformations that have varying degrees of reactivity. This conclusion does not contradict the observations that polymerases exist in different conformations during correct and mispair formation.^{3, 15} The spectroscopic and structural studies may have identified specific complexes along a continuum of conformations that the polymerase can adopt.

One aspect complicating the interpretation of the results is that the k_{pol} is a complex parameter; it is a function of kinetic constants leading up to and including the chemistry step. The rate limiting step for all reactions, except correct base pair formation, is phosphodiester bond formation. However, for correct base pair formation, the rate limiting step is the conformational changes surrounding phosphoryl transfer. Thus, for all reactions, except the correct base pair formation, the $k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{3\text{DG}}$ represents the effect of the 3DG substitution on phosphodiester bond formation. For correct base pair formation, the 3DG substitution changes the rate limiting step from the conformational change to the bond formation step. Thus, $k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{3\text{DG}}$ underestimates the effect of the 3DG substitution on the reduction in the rate of phosphodiester bond formation. The rate of phosphodiester bond formation is unknown, but is estimated to be $> 1000 \text{ s}^{-1}$ by pre-steady-state kinetics and computer modelling.¹⁹ Thus, the effect of the 3DG substitution on phosphoryl transfer during correct base pair formation is ~ 10 -fold higher than $k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{3\text{DG}}$. This correction would place correct base pairs data near the upside-down magenta triangle in Figure 4. This complication does not necessarily affect interpretation of the results.

In conclusion, we have utilized atomic substitution of the DNA to probe the role of Arg668 in catalysis of DNA replication by KF. We utilized carcinogen-modified DNA to extend the reactivity of undamaged DNA. We found that, the presence of N3 at the primer terminus

plays a minor role in dNTP binding and a major role in phosphoryl transfer. The linear relationship between $\log(k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{3\text{DG}})$ and $\log(k_{\text{pol}}^{\text{G}})$ indicates that the mechanism by which Arg668 helps to catalyze phosphoryl transfer is the same with the various substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BF	<i>Bacillus stearothermophilis</i> DNA polymerase large fragment
BP	benzo[a]pyrene
BPDE	7 <i>R</i> ,8 <i>S</i> -dihydrodiol-9 <i>R</i> ,10 <i>R</i> -epoxy-7,8,9,10-tetrahydrobenzo[<i>a</i>]pyrene
BSA	bovine serum albumin
3DG	3-deazaguanine
KF	Klenow fragment of <i>E. coli</i> DNA polymerase I with the proofreading exonuclease inactivated
N²-BP-G	N ² -[(7 <i>S</i> ,8 <i>R</i> ,9 <i>S</i> ,10 <i>R</i>)-7,8,9,10-tetrahydro-8,9,10-trihydroxybenzo[<i>a</i>]pyren-7-yl]-guanine
O⁶mG	O ⁶ -methyl-2'-deoxyguanosine
oxoG	8-oxo-7,8-dihydro-2'-deoxyguanosine
O²-POB-T	O ² -[4-(3-pyridyl)-4-oxobutyl]thymine
T7 pol	T7 DNA polymerase

References

1. Kunkel TA. DNA Replication Fidelity. *J Biol Chem.* 2004; 279:16895–16898. [PubMed: 14988392]
2. Watson JD, Crick FH. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature.* 1953; 171:737–738. [PubMed: 13054692]
3. Johnson KA. The kinetic and chemical mechanism of high-fidelity DNA polymerases. *Biochim Biophys Acta.* 2010; 1804:1041–1048. [PubMed: 20079883]
4. Hunter WN, Brown T, Knele G, Anand NN, Rabinovich D, Kennard O. The structure of guanosine-thymidine mismatches in B-DNA at 2.5-Å resolution. *J Biol Chem.* 1987; 262:9962–9970. [PubMed: 3611072]
5. Hunter WN, Brown T, Anand NN, Kennard O. Structure of an adenine-cytosine base pair in DNA and its implications for mismatch repair. *Nature.* 1986; 320:552–555. [PubMed: 3960137]
6. Brown T, Kennard O. Structural basis of DNA mutagenesis. *Curr Opin Struct Biol.* 1992; 2:354–360.

7. Petruska J, Goodman MF, Boosalis MS, Sowers LC, Cheong C, Tinoco I Jr. Comparison between DNA melting thermodynamics and DNA polymerase fidelity. *Proc Natl Acad Sci U S A*. 1988; 85:6252–6256. [PubMed: 3413095]
8. Echols H, Goodman MF. Fidelity mechanisms in DNA replication. *Annu Rev Biochem*. 1991; 60:477–511. [PubMed: 1883202]
9. Oertell K, Harcourt EM, Mohsen MG, Petruska J, Kool ET, Goodman MF. Kinetic selection vs. free energy of DNA base pairing in control of polymerase fidelity. *Proc Natl Acad Sci U S A*. 2016; 113:E2277–E2285. [PubMed: 27044101]
10. Kunkel TA, Bebenek K. DNA Replication fidelity. *Annu Rev Biochem*. 2000; 69:497–529. [PubMed: 10966467]
11. Kool ET. Active site tightness and substrate fit in DNA replication. *Annu Rev Biochem*. 2002; 71:191–219. [PubMed: 12045095]
12. Moran S, Ren RXF, Rumney SI, Kool ET. Difluorotoluene, a nonpolar isostere for thymine, codes specifically and efficiently for adenine in DNA replication. *J Am Chem Soc*. 1997; 119:2056–2057. [PubMed: 20737028]
13. Morales JC, Kool ET. Efficient replication between non-hydrogen-bonded nucleoside shape analogs. *Nat Struct Biol*. 1998; 5:950–954. [PubMed: 9808038]
14. Potapova O, Chan C, DeLucia AM, Helquist SA, Kool ET, Grindley NDF, Joyce CM. DNA polymerase catalysis in the absence of Watson-Crick hydrogen bonds: analysis by single-turnover kinetics. *Biochemistry*. 2005; 45:890–898.
15. Tsai YC, Johnson KA. A new paradigm for DNA polymerase specificity. *Biochemistry*. 2006; 45:9675–9687. [PubMed: 16893169]
16. Ollis DL, Brick P, Hamlin R, Xuong NG, Steitz TA. Structure of large fragment of *Escherichia coli* DNA polymerase I complexed with dTMP. *Nature*. 1985; 313:762–766. [PubMed: 3883192]
17. Patel SS, Wong I, Johnson KA. Pre-steady-state kinetic analysis of processive DNA replicative including complete characterization of an exonuclease-deficient mutant. *Biochemistry*. 1991; 30:511–525. [PubMed: 1846298]
18. Wong I, Patel SS, Johnson KA. An induced-fit kinetic mechanism for DNA replication fidelity: direct measurement by single-turnover kinetics. *Biochemistry*. 1991; 30:526–537. [PubMed: 1846299]
19. Kuchta RD, Mizrahi V, Benkovic PA, Johnson KA, Benkovic SJ. Kinetic mechanism of DNA polymerase I (Klenow). *Biochemistry*. 1987; 26:8410–8417. [PubMed: 3327522]
20. Kuchta RD, Benkovic P, Benkovic SJ. Kinetic mechanism whereby DNA polymerase I (Klenow) replicates DNA with high fidelity. *Biochemistry*. 1988; 27:6716–6725. [PubMed: 3058205]
21. Eger BT, Benkovic SJ. Minimal kinetic mechanism for misincorporation by DNA polymerase I (Klenow fragment). *Biochemistry*. 1992; 31:9227–9236. [PubMed: 1327109]
22. Gowda AS, Moldovan GL, Spratt TE. Human DNA polymerase ϵ catalyzes correct and incorrect DNA synthesis with high catalytic efficiency. *J Biol Chem*. 2015; 290:16292–16303. [PubMed: 25963146]
23. Kiefer JR, Mao C, Braman JC, Beese LS. Visualizing DNA replication in a catalytically active *Bacillus* DNA polymerase crystal. *Nature*. 1998; 391:304–307. [PubMed: 9440698]
24. Li Y, Korolev S, Waksman G. Crystal structures of open and closed forms of binary and ternary complexes of the large fragment of *Thermus aquaticus* DNA polymerase I: structural basis for nucleotide incorporation. *EMBO J*. 1998; 17:7514–7525. [PubMed: 9857206]
25. Doubie S, Tabor S, Long AM, Richardson CC, Ellenberger T. Crystal structure of a bacteriophage T7 DNA replication complex at 2.2 Å resolution. *Nature*. 1998; 391:251–258. [PubMed: 9440688]
26. Szymanski MR, Kuznetsov VB, Shumate C, Meng Q, Lee YS, Patel G, Patel S, Yin YW. Structural basis for processivity and antiviral drug toxicity in human mitochondrial DNA replicase. *EMBO J*. 2015; 34:1959–1970. [PubMed: 26056153]
27. Rothwell PJ, Mitaksov V, Waksman G. Motions of the fingers subdomain of KlenTaq1 are fast and not rate limiting: implications for the molecular basis of fidelity in DNA polymerases. *Mol Cell*. 2005; 19:345–355. [PubMed: 16061181]

28. Luo G, Wang M, Konigsberg WH, Xie XS. Single-molecule and ensemble fluorescence assays for a functionally important conformational change in T7 DNA polymerase. *Proc Natl Acad Sci USA*. 2007; 104:12610–12615. [PubMed: 17640918]
29. Santoso Y, Joyce CM, Potapova O, Le Reste L, Hohlbein J, Torella JP, Grindley ND, Kapanidis AN. Conformational transitions in DNA polymerase I revealed by single-molecule FRET. *Proc Natl Acad Sci U S A*. 2010; 107:715–720. [PubMed: 20080740]
30. Joyce CM, Benkovic SJ. DNA polymerase fidelity: kinetics, structure, and checkpoints. *Biochemistry*. 2004; 43:14317–14324. [PubMed: 15533035]
31. Hohlbein J, Aigrain L, Craggs TD, Bermek O, Potapova O, Shoolizadeh P, Grindley ND, Joyce CM, Kapanidis AN. Conformational landscapes of DNA polymerase I and mutator derivatives establish fidelity checkpoints for nucleotide insertion. *Nat Commun*. 2013; 4:2131. [PubMed: 23831915]
32. Wu EY, Beese LS. The structure of a high fidelity DNA polymerase bound to a mismatched nucleotide reveals an “Ajar” intermediate conformation in the nucleotide selection mechanism. *J Biol Chem*. 2011; 286:19758–19767. [PubMed: 21454515]
33. Wang W, Hellinga HW, Beese LS. Structural evidence for the rare tastier hypothesis of spontaneous mutagenesis. *Proc Natl Acad Sci U S A*. 2011; 108:17644–17648. [PubMed: 22006298]
34. Trostler M, Delier A, Beckman J, Urban M, Patro JN, Spratt TE, Beese LS, Kuchta RD. Discrimination between right and wrong purine dNTPs by DNA polymerase I from *Bacillus stearothermophilus*. *Biochemistry*. 2009; 48:4633–4641. [PubMed: 19348507]
35. Beckman J, Kincaid K, Hocek M, Spratt T, Engels J, Cosstick R, Kuchta RD. Human DNA polymerase alpha uses a combination of positive and negative selectivity to polymerize purine dNTPs with high fidelity. *Biochemistry*. 2007; 46:448–460. [PubMed: 17209555]
36. Arana ME, Takata K-i, Garcia-Diaz M, Wood RD, Kunkel TA. A unique error signature for human DNA polymerase γ . *DNA Repair*. 2007; 6:213–223. [PubMed: 17118716]
37. Sohl CD, Szymanski MR, Mislak AC, Shumate CK, Amiralaie S, Schinazi RF, Anderson KS, Yin YW. Probing the structural and molecular basis of nucleotide selectivity by human mitochondrial DNA polymerase γ . *Proc Natl Acad Sci U S A*. 2015; 112:8596–8601. [PubMed: 26124101]
38. Seeman NC, Rosenberg JM, Rich A. Sequence-specific recognition of double helical nucleic acids by proteins. *Proc Natl Acad Sci U S A*. 1976; 73:804–808. [PubMed: 1062791]
39. Polesky AH, Steitz TA, Grindley NDF, Joyce CM. Identification of residues critical for the polymerase activity of the Klenow fragment of DNA polymerase I from *Escherichia coli*. *J Biol Chem*. 1990; 265:14579–14591. [PubMed: 2201688]
40. Polesky AH, Dahlberg ME, Benkovic SJ, Grindley NDF, Joyce CM. Side chains involved in catalysis of polymerase reaction of DNA polymerase I from *Escherichia coli*. *J Biol Chem*. 1992; 267:8417–8428. [PubMed: 1569092]
41. Minnick DT, Bebenek K, Osheroff WP, Turner RM Jr, Astatke M, Liu L, Kunkel Ta, Joyce CM. Side chains that influence fidelity at the polymerase active site of *Escherichia coli* DNA polymerase I (Klenow fragment). *J Biol Chem*. 1999; 274:3067–3075. [PubMed: 9915846]
42. Meyer AS, Blandino M, Spratt TE. *E. coli* DNA polymerase I (Klenow fragment) uses a hydrogen bonding fork from Arg668 to the primer terminus and incoming deoxynucleotide triphosphate to catalyze DNA replication. *J Biol Chem*. 2004; 279:33043–33046. [PubMed: 15210707]
43. McCain MD, Meyer AS, Glekas A, Spratt TE. Fidelity of mispair formation and mispair extension is dependent on the interaction between the minor groove of the primer terminus and Arg668 of DNA Polymerase I of *E. coli*. *Biochemistry*. 2005; 44:5647–5659. [PubMed: 15823023]
44. Dunn, DB., Hall, RH. *Handbook of Biochemistry and Molecular Biology*. Fasman, GD., editor. CRC Press; Boca Raton: 1986. p. 65-215.
45. Spratt TE. Identification of hydrogen bonds between *Escherichia coli* DNA polymerase I (Klenow fragment) and the minor groove of DNA by amino acid substitution of the polymerase and atomic substitution of the DNA. *Biochemistry*. 2001; 40:2647–2652. [PubMed: 11258875]
46. Krishnegowda G, Sharma AK, Krzeminski J, Gowda ASP, Lin JM, Desai D, Spratt TE, Amin S. Facile syntheses of O^2 -[4-(3-pyridyl-4-oxobut-1-yl)]thymidine, the major adduct formed by tobacco specific nitrosamine 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in vivo, and

Its site-specifically adducted oligodeoxynucleotides. *Chem Res Toxicol*. 2011; 24:960–967. [PubMed: 21524094]

47. Gowda ASP, Krzeminski J, Amin S, Suo Z, Spratt TE. Mutagenic replication of *N*²-deoxyguanosine benzo[*a*]pyrene adducts by *Escherichia coli* DNA polymerase I and *Sulfolobus solfataricus* DNA polymerase IV. *Chem Res Toxicol*. 2017; 30:1168–1176. [PubMed: 28402640]
48. Gowda ASP, Spratt TE. DNA polymerases η and ζ combine to bypass O²-[4-(3-pyridyl)-4-oxobutyl]thymine, a DNA adduct formed from tobacco carcinogens. *Chem Res Toxicol*. 2016; 29:303–316. [PubMed: 26868090]
49. Fiala KA, Suo Z. Mechanism of DNA polymerization catalyzed by *Sulfolobus solfataricus* P2 DNA polymerase IV. *Biochemistry*. 2004; 43:2116–2125. [PubMed: 14967051]
50. Dahlberg ME, Benkovic SJ. Kinetic mechanism of DNA polymerase I (Klenow fragment): identification of a second conformational change and evaluation of the internal equilibrium constant. *Biochemistry*. 1991; 30:4835–4843. [PubMed: 1645180]
51. Eger BT, Kuchta RD, Carroll SS, Benkovic PA, Dahlberg ME, Joyce CM, Benkovic SJ. Mechanism of DNA replication fidelity for three mutants of DNA polymerase I: Klenow fragment KF(exo+), KF(polA5), and KF(exo-). *Biochemistry*. 1991; 30:1441–1448. [PubMed: 1991125]
52. Dosanjh MK, Galeros G, Goodman MF, Singer B. Kinetics of extension of O 6 -methylguanine paired with cytosine or thymine in defined oligonucleotide sequences. *Biochemistry*. 1991; 30:11595–11599. [PubMed: 1747377]
53. Woodside AM, Guengerich FP. Effect of the O⁶-substituent on misincorporation kinetics catalyzed by DNA polymerases at O⁶-methylguanine and O⁶-benzylguanine. *Biochemistry*. 2002; 41:1027–1038. [PubMed: 11790127]
54. Eoff RL, Irimia A, Egli M, Guengerich FP. *Sulfolobus solfataricus* DNA polymerase Dpo4 is partially inhibited by “wobble” pairing between O⁶-methylguanine and cytosine, but accurate bypass is preferred. *J Biol Chem*. 2007; 282:1456–1467. [PubMed: 17105728]
55. Loveless A. Possible relevance or O-6 alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. *Nature*. 1969; 223:206–207. [PubMed: 5791738]
56. Swann PF. Why do O⁶-alkylguanine and O⁴-alkylthymine miscode? The relationship between the structure of DNA containing O⁶-alkylguanine and O⁴-alkylthymine and the mutagenic properties of these bases. *Mutat Res*. 1990; 233:81–94. [PubMed: 2233815]
57. Leonard GA, Thomson J, Watson WP, Brown T. High-resolution structure of a mutagenic lesion in DNA. *Proc Natl Acad Sci USA*. 1990; 87:9573–9676. [PubMed: 2263612]
58. Williams LD, Shaw BR. Protonated base pairs explain the ambiguous pairing properties of O 6 -methylguanine. *Proc Natl Acad Sci USA*. 1987; 84:1779–1783. [PubMed: 3470757]
59. Patel DJ, Shapiro L, Kozłowski SA, Gaffney BL, Jones RA. Structural studies of the O6meG-C interaction in the d(C-G-C-G-A-A-T-T-C-O6meG-C-G) duplex. *Biochemistry*. 1986; 25:1027–1036. [PubMed: 3964658]
60. Patel DJ, Shapiro L, Kozłowski SA, Gaffney BL, Jones RA. Structural studies of the O6meG-T interaction in the d(C-G-T-G-A-A-T-T-C-O6meG-C-G) duplex. *Biochemistry*. 1986; 25:1036–1042. [PubMed: 3964659]
61. Li, BF., Swann, PF., Kalnik, MW., Kouchakdjian, M., Patel, DJ. NMR solution studies of colvalent carcinogenic lesions in DNA: O⁶-methylguanosine, O⁶-ethylguanosine and O⁴-methylthymidine. In: Jaroszewski, JW., editor. *NMR Spectroscopy in Drug Research*. Copenhagen; 1988. p. 309-340.
62. Spratt TE, Levy DE. Structure of the hydrogen bonding complex of O⁶-methylguanine with cytosine and thymine during DNA replication. *Nucleic Acids Res*. 1997; 25:3354–3361. [PubMed: 9241252]
63. Warren JJ, Forsberg LJ, Beese LS. The structural basis for the mutagenicity of O⁶-methyl-guanine lesions. *Proc Natl Acad Sci USA*. 2006; 103:19701–19706. [PubMed: 17179038]
64. Tan HB, Swann PF, Chance EM. Kinetic analysis of the coding properties of O6-methylguanine in DNA: the crucial role of the conformation of the phosphodiester bond. *Biochemistry*. 1994; 33:5335–5346. [PubMed: 8172907]

65. Woodside AM, Guengerich FP. Misincorporation and stalling at O⁶-methylguanine and O⁶-benzylguanine: evidence for inactive polymerase complexes. *Biochemistry*. 2002; 41:1039–1050. [PubMed: 11790128]
66. Lowe LG, Guengerich FP. Steady-state and pre-steady-state kinetic analysis of dNTP insertion opposite 8-oxo-7,8-dihydroguanine by *Escherichia coli* polymerase I *exo* - and II *exo* - *Biochemistry*. 1996; 35:9840–9849. [PubMed: 8703958]
67. Furge LL, Guengerich FP. Analysis of nucleotide insertion and extension at 8-oxo-7,8-dihydroguanine by replicative T7 polymerase *exo* - and human immunodeficiency virus-1 reverse transcriptase using steady-state and pre-steady-state kinetics. *Biochemistry*. 1997; 36:6475–6487. [PubMed: 9174365]
68. Zhang Y, Yuan F, Wu X, Wang MR, Taylor JS, Geacintov NE, Wang Z. Error-free and error-prone lesion bypass by human DNA polymerase kappa in vitro. *Nucleic Acids Res*. 2000; 28:4138–4146. [PubMed: 11058110]
69. Miller H, Prasad R, Wilson SH, Johnson SH, Grollman AP. 8-OxodGTP incorporation by DNA polymerase beta is modified by active-site residue Asn279. *Biochemistry*. 2000; 39:1029–1033. [PubMed: 10653647]
70. Carlson KD, Washington MT. Mechanism of efficient and accurate nucleotide incorporation opposite 7,8-dihydro-8-oxoguanine by *Saccharomyces cerevisiae* DNA polymerase eta. *Mol Cell Biol*. 2005; 25:2169–2176. [PubMed: 15743815]
71. Zang H, Irimia A, Choi JY, Angel KC, Loukachevitch LV, Egli M, Guengerich FP. Efficient and high fidelity incorporation of dCTP opposite 7,8-dihydro-8-oxodeoxyguanosine by *Sulfolobus solfataricus* DNA polymerase Dpo4. *J Biol Chem*. 2006; 281:2358–2372. [PubMed: 16306039]
72. Shibutani S, Takeshita M, Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation base 8-oxodG. *Nature*. 1991; 349:431–434. [PubMed: 1992344]
73. Briebe LG, Eichman BF, Kokoska RJ, Doublet S, Kunkel TA, Ellenberger T. Structural basis for the dual coding potential of 8-oxoguanosine by a high-fidelity DNA polymerase. *EMBO J*. 2004; 23:3452–3461. [PubMed: 15297882]
74. Briebe LG, Kokoska RJ, Bebenek K, Kunkel TA, Ellenberger T. A lysine residue in the fingers subdomain of T7 DNA polymerase modulates the miscoding potential of 8-oxo-7,8-dihydroguanosine. *Structure (London, England : 1993)*. 2005; 13:1653–1659.
75. Dipple, A., Moschel, RC., Bigger, CA. Polynuclear aromatic carcinogens. In: Searle, CE., editor. *Chemical Carcinogens (ACS Monograph 182)*. American Chemical Society; Washington DE: 1984. p. 41-163.
76. Avkin S, Goldsmith M, Velasco-Miguel S, Geacintov N, Friedberg EC, Livneh Z. Quantitative analysis of translesion DNA synthesis across a benzo[a]pyrene-guanine adduct in mammalian cells: the role of DNA polymerase kappa. *J Biol Chem*. 2004; 279:53298–53305. [PubMed: 15475561]
77. Hsu GW, Huang X, Luneva NP, Geacintov NE, Beese LS. Structure of a high-fidelity DNA polymerase bound to a benzo[a]pyrene adduct that blocks replication. *J Biol Chem*. 2005; 280:3764–3770. [PubMed: 15548515]
78. Perlow RA, Broyde S. Evading the proofreading machinery of a replicative DNA polymerase: induction of a mutation by an environmental carcinogen. *J Mol Biol*. 2001; 309:519–536. [PubMed: 11371169]
79. Perlow RA, Broyde S. Extending the understanding of mutagenicity: structural insights into primer-extension past a benzo[a]pyrene diol epoxide-DNA adduct. *J Mol Biol*. 2003; 327:797–818. [PubMed: 12654264]
80. Xu P, Oum L, Beese LS, Geacintov NE, Broyde S. Following an environmental carcinogen N2-dG adduct through replication: elucidating blockage and bypass in a high-fidelity DNA polymerase. *Nucleic Acids Res*. 2007; 35:4275–4288. [PubMed: 17576677]
81. Lao Y, Yu N, Kassie F, Villalta PW, Hecht SS. Formation and accumulation of pyridyloxobutyl DNA adducts in F344 rats chronically treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and enantiomers of its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol. *Chem Res Toxicol*. 2007; 20:235–245. [PubMed: 17305407]

82. Lao Y, Yu N, Kassie F, Villalta PW, Hecht SS. Analysis of pyridyloxobutyl DNA adducts in F344 rats chronically treated with (R)- and (S)-N'-nitrosornicotine. *Chem Res Toxicol*. 2007; 20:246–256. [PubMed: 17305408]
83. Weerasooriya S, Jasti VP, Bose A, Spratt TE, Basu AK. Roles of translesion synthesis DNA polymerases in the potent mutagenicity of tobacco-specific nitrosamine-derived O-alkylthymidines in human cells. *DNA Repair (Amst)*. 2015; 35:63–70. [PubMed: 26460881]
84. Gowda ASP, Krishnegowda G, Suo Z, Amin S, Spratt TE. Low fidelity bypass of O²-(3-pyridyl)-4-oxobutylthymine, the most persistent bulky adduct produced by the tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by model DNA polymerases. *Chem Res Toxicol*. 2012; 25:1195–1202. [PubMed: 22533615]
85. Brody RS, Frey PA. Unambiguous determination of the stereochemistry of nucleotidyl transfer catalyzed by DNA polymerase I from *Escherichia coli*. *Biochemistry*. 1981; 20:1245–1252. [PubMed: 7013792]
86. Batra VK, Perera L, Lin P, Shock DD, Beard WA, Pedersen LC, Pedersen LG, Wilson SH. Amino acid substitution in the active site of DNA polymerase β explains the energy barrier of the nucleotidyl transfer reaction. *J Am Chem Soc*. 2013; 135:8078–8088. [PubMed: 23647366]
87. Cisneros GA, Perera L, García-Díaz M, Bebenek K, Kunkel TA, Pedersen LG. Catalytic mechanism of human DNA polymerase λ with Mg²⁺ and Mn²⁺ from ab initio quantum mechanical/molecular mechanical studies. *DNA Repair*. 2008; 7:1824–1834. [PubMed: 18692600]
88. Lin P, Pedersen LC, Batra VK, Beard WA, Wilson SH, Pedersen LG. Energy analysis of chemistry for correct insertion by DNA polymerase β . *Proc Natl Acad Sci USA*. 2006; 103:13294–13299. [PubMed: 16938895]
89. Lee YS, Gao Y, Yang W. How a homolog of high-fidelity replicases conducts mutagenic DNA synthesis. *Nat Struct Mol Biol*. 2015; 22:298–303. [PubMed: 25775266]
90. Jencks, WP. Catalysis in chemistry and enzymology. McGraw-Hill Book Company; New York: 1969.
91. Hunter WN, Brown T, Kneale G, Anand NN, Rabinovich D, Kennard O. The structure of guanosine-thymidine mismatches in B-DNA at 2.5-Å resolution. *J Biol Chem*. 1987; 262:9962–9970. [PubMed: 3611072]
92. Allawi HT, SantaLucia J. NMR solution structure of a DNA dodecamer containing single G-T mismatches. *Nucleic Acids Res*. 1998; 26:4925–4934. [PubMed: 9776755]
93. Johnson SJ, Beese LS. Structures of mismatch replication errors observed in a DNA polymerase. *Cell*. 2004; 116:803–816. [PubMed: 15035983]
94. Isaacs RJ, Rayens WS, Spielmann HP. Structural differences in the NOE-derived structure of G-T mismatched DNA relative to normal DNA are correlated with differences in (13)C relaxation-based internal dynamics. *J Mol Biol*. 2002; 319:191–207. [PubMed: 12051946]
95. Alvarez-Salgado F, Desvaux H, Boulard Y. NMR assessment of the global shape of a non-labelled DNA dodecamer containing a tandem of G-T mismatches. *Magn Reson Chem*. 2006; 44:1081–1089. [PubMed: 16972306]
96. Bebenek K, Pedersen LC, Kunkel TA. Replication infidelity via a mismatch with Watson–Crick geometry. *Proc Natl Acad Sci U S A*. 2011; 108:1862–1867. [PubMed: 21233421]
97. Sowers LC, Goodman MF, Eritja R, Kaplan B, Fazakerley GV. Ionized and wobble base-pairing for bromouracil-guanine in equilibrium under physiological conditions. *J Mol Biol*. 1989; 205:437–447. [PubMed: 2538629]
98. Sowers LC, Eritja R, Kaplan BE, Goodman MF, Fazakerley GV. Equilibrium between a wobble and ionized base pair formed between fluorouracil and guanine in DNA as studied by proton and fluorine NMR. *J Biol Chem*. 1988; 263:14794–14801. [PubMed: 3170564]
99. Koag M-C, Lee S. Metal-dependent conformational activation explains highly promutagenic replication across O⁶-methylguanine by human DNA polymerase β . *J Am Chem Soc*. 2014; 136:5709–5721. [PubMed: 24694247]

Figure 1. Representations of the Arg-fork in KF. A Three dimensional view of the Arg-fork.⁴² B. Schematic view of how changing X from G to 3DG will affect the Arg-fork

Figure 2.
Structures of the nucleotide analogs used in this study.

Figure 3.

Evaluation of rate limiting step with 3DG at primer terminus. Thio-effect with G (A) or 3DG (B) at the primer terminus. The reactions contained 200 nM KF⁻, 70 nM DNA, 100 μ M dCTP or dCTP α S. Pulse-chase experiment during the incorporation of dCTP opposite dG with G (C) and 3DG (D) at the primer terminus. The reactions contained 75 nM KF⁻, 150 nM DNA, and 2.5 μ M [α -³²P] dCTP. Differences in EDTA versus HCl quench during the incorporation of dCTP opposite dG with G (E) and 3DG (F) at the primer terminus or the incorporation of dUTP (G) and dcUTP (H) opposite dA. The reactions contained 75 nM KF⁻, 150 nM DNA, and 100 μ M dNTP. The data points are the mean $\pm$ standard deviation of three experiments. The lines are best fit to the burst or first-order equations.

Figure 4.

Effect of breaking Arg-fork on k_{pol} . Relationship of $k_{\text{pol}}^{\text{G}}/k_{\text{pol}}^{\text{3DG}}$ to $k_{\text{pol}}^{\text{G}}$. The incoming dNTP is represented by color, and the template by shape. The blue line is the least-square linear fit to the incorporation of dTTP opposite G, O⁶mG, and oxoG. The black line is the best-fit to the other points. The upside-down magenta triangle represents the estimated position of the effect of the 3DG substitution on the rate of phosphodiester bond formation.

Figure 5.

Potential base pair structures. (A) Watson-Crick 8mG/T; (B) distorted Watson-Crick 8mG/T; (C) distorted Watson-Crick 8mG/C; (D) wobble G/T (E) wobble G/T with bifurcated hydrogen bonds. (F) Watson-Crick G/T with minor tautomer,

Scheme 1.

Mechanisms of DNA polymerases^a

a, E represents the polymerase, E*, the polymerase in the closed configuration, D_n the DNA substrate with primer of length n, R, the correct dNTP, W, the wrong dNTP, P, pyrophosphate, D_{n+R} , the DNA product in which the right dNTP was incorporated, D_{n+W} the DNA product in which the wrong dNTP was incorporated