Hydrogen Bonding of 7,8-Dihydro-8-oxodeoxyguanosine with a Charged Residue in the Little Finger Domain Determines Miscoding Events in *Sulfolobus solfataricus* DNA Polymerase Dpo4*[^2]*

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*Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4) has been shown to catalyze bypass of 7,8-dihydro-8-oxodeoxyguanosine (8-oxoG) in a highly efficient and relatively accurate manner. Crystal structures have revealed a potential role for Arg[^332] in stabilizing the anti conformation of the 8-oxoG template base by means of a hydrogen bond or ion-dipole pair, which results in an increased enzymatic efficiency for dCTP insertion and makes formation of a Hoogsteen pair between 8-oxoG and dATP less favorable. Site-directed mutagenesis was used to replace Arg[^332] with Ala, Glu, Leu, or His in order to probe the importance of Arg[^332] in accurate and efficient bypass of 8-oxoG. The double mutant Ala[^331]Ala[^332] was also prepared to address the contribution of Arg[^331]. Transient-state kinetic results suggest that Glu[^332] retains fidelity against bypass of 8-oxoG that is similar to wild type Dpo4, a result that was confirmed by tandem mass spectrometric analysis of full-length extension products. A crystal structure of the Dpo4 Glu[^332] mutant and 8-oxoG:C pair revealed water-mediated hydrogen bonds between Glu[^332] and the O-8 atom of 8-oxoG. The space normally occupied by Arg[^332] side chain is empty in the crystal structures of the Ala[^332] mutant. Two other crystal structures show that a Hoogsteen base pair is formed between 8-oxoG and A in the active site of both Glu[^332] and Ala[^332] mutants. These results support the view that a bond between Arg[^332] and 8-oxoG plays a role in determining the fidelity and efficiency of Dpo4-catalyzed bypass of the lesion.

Enzyme-catalyzed reactions are fundamental in biology (1–3). Certain elements of an enzymatic mechanism may be obvious once elucidated (i.e. required catalytic residues, prosthethic groups, the presence or absence of cofactors), and the relevance for function is readily discerned. In other instances, features of interest may be difficult to identify in the multitude of non-essential elements that often comprise enzyme-substrate interactions (4, 5). For example, nucleotide selection and subsequent incorporation by DNA polymerases have been described in great detail (6, 7), and the chemical components necessary for phosphoryl transfer are conserved across all domains of life and across all polymerase subfamilies. Yet, in the course of analyzing different polymerases it has become apparent that not all of these enzymes use the same mechanism(s) to define substrate specificity, a vital aspect of polymerase function (8).

The Y-family DNA polymerases appear to present a case of relaxed substrate selection, but the less accurate mode of copying template DNA is supplanted by the greater propensity of Y-family polymerases to effectively utilize damaged DNA as a substrate (9–12). Indeed, the ubiquitous nature of DNA damage, both endogenous and exogenous in origin, makes it necessary for the replisome to have some means of bypassing covalently modified DNA (13). Some general features unique to Y-family polymerases that are relevant to translesion DNA synthesis include an active site that leaves the newly formed base pair relatively unconstrained by protein-DNA interactions and employment of an additional domain termed the “little finger” or palm-associated domain (PAD), which has important contacts with the template DNA near the active site (14, 15). Other mechanistic features of the Y-family that likely contribute to effective partitioning between low and high-fidelity polymerases include low processivity, relatively slow forward rates of polymerization (k[^pol]), and the requirement for high dNTP concentrations to achieve maximum catalytic rates (i.e. “high” K[^p]dNTP) (16–19). Previous work from our group has shown that a model Y-family polymerase, Dpo4 from *Sulfolobus solfataricus* P2, is able to bypass 8-oxoG[^3], a major lesion arising from

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[^4]: The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S31 and Tables S1–S6 detailing a large portion of the data presented in the text. The atomic coordinates and structure factors (codes 2uvw, 2uvv, 2uvu, 2uvr) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[^5]: The abbreviations used are: 8-oxoG, 7,8-dihydro-8-oxodeoxyguanosine; Dpo4, DNA polymerase IV; CID, collision-induced dissociation; DTT, dithiothreitol; ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; pol, (DNA) polymerase; pol T7, bacteriophage pol T7 (exonuclease-deficient); WT, wild type.
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oxidative stress, in a highly accurate and efficient manner (20). The kinetic parameters and LC-MS analysis of extension products indicated that Dpo4 was ~20-fold more efficient at insertion of dCTP opposite 8-oxoG relative to dATP insertion. X-ray crystal structures revealed that the 8-oxoG:A pair was in the synanti configuration, which allows A to form a Hoogsteen pair with 8-oxoG. Conversely, the 8-oxoG:C pair was in the Watson-Crick geometry and such a configuration appeared to be stabilized by either a hydrogen bond or an ion-dipole pair between the O-8 atom and the side chain of Arg<sup>332</sup>. The importance of such an interaction was further confirmed by separate studies that observed a water-mediated hydrogen bond between Arg<sup>332</sup> and 8-oxoG (21).

Thermodynamically and geometrically the 8-oxoG:A pair is similar to a T:A pair. In isolated oligonucleotides the 8-oxoG:A pair decreases the T<sub>m</sub> by 5.5 °C relative to T:A, and the T<sub>m</sub> for the corresponding 8-oxoG:C pair is 16.9 °C lower than a G:C pair (22). The question then arises as to why Dpo4 retains high efficiency and fidelity during bypass of 8-oxoG. The role of Arg<sup>332</sup> in nucleotide selection during Dpo4-catalyzed bypass of 8-oxoG was investigated using a series of mutant enzymes. In an effort to determine what factors contribute to the stabilization of the 8-oxoG:C pair, site-directed mutagenesis was used to alter different chemical aspects of the Arg<sup>332</sup> side chain, including steric occupancy and hydrogen bonding potential. Transient-state kinetic and LC-MS/MS analyses were then combined with x-ray crystallographic studies to compare mutant-catalyzed bypass of 8-oxoG with wild type Dpo4.

**EXPERIMENTAL PROCEDURES**

*MATERIALS—Site-directed mutagenesis was performed using synthetic oligonucleotide primers and a QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Wild type Dpo4 and all of the mutant enzymes were expressed in *Escherichia coli* and purified to electrophoretic homogeneity as described previously (23). All unlabeled dNTPs were obtained from Amersham Biosciences, and [γ<sup>32</sup>P]ATP was purchased from PerkinElmer Life Sciences. All oligonucleotides used in this work were synthesized by Midland Certified Reagent Co. (Midland, TX) and purified using high performance liquid chromatography by the manufacturer, with analysis by matrix-assisted laser desorption time-of-flight MS. The 13-base primer sequences used in the kinetic and mass spectral analyses was 5′-GAATCCCTCCC-3′. The 14-base primer sequences used in the insertion experiments was 5′-TCATGGAAATCTCTCCCCC-3′, where X = G or 8-oxoG, as indicated. The DNA control template sequence used in the full-length extension assay (Fig. 1B) was 5′-TCATGGAAATCTCCTCCCCC-3′.

*Full-length Extension Assay—A 32P-labeled primer was annealed to an unmodified or adducted template oligonucleotide. Each reaction was initiated by adding dNTP-Mg<sup>2+</sup> (each dNTP at 250 μM and 5 mM MgCl<sub>2</sub>) solution to a preincubated Dpo4-DNA complex (100 nM Dpo4 and 200 nM DNA).

The reaction was carried out at 37 °C in 50 mM Tris-HCl (pH 7.4) buffer containing 50 mM NaCl, 5 mM DTT, 100 μg μl<sup>−1</sup> bovine serum albumin, and 5% (v/v) glycerol. At the indicated time, 5-μl aliquots were quenched with 50 μl of 500 mM EDTA (pH 9.0). The samples were then mixed with 100 μl of a 95% formamide/20 mM EDTA solution and separated on a 20% polyacrylamide (w/v)/7 m urea gel. Products were visualized and quantified using a phosphorimaging screen and Quantity One<sup>TM</sup> software, respectively (Bio-Rad). Formation of an 8-oxoG-containing product from a 13-base primer was quantified by fitting the data to Equation 1,

\[
f_{1B}(t) = A \left(1 - \sum_{i=1}^{n} \left(\frac{(k_{obs}t)^{i-1}}{(r-1)!} \right) e^{-k_{act}t}\right)
\]

where A = amount of product formed during the first binding event between Dpo4 and DNA, k<sub>obs</sub> = an observed rate constant defining nucleotide incorporation, n = number of incorporation events required to observe product formation, and t = time. All statistical values given indicate S.E.

*Transient-state Kinetics—All pre-steady-state experiments were performed using a KinTek RQF-3 model chemical quench-flow apparatus (KinTek Corp., Austin, TX) with 50 mM Tris-HCl (pH 7.4) buffer in the drive syringes. All RQF experiments were carried out at 37 °C in a buffer containing 50 mM Tris-HCl (pH 7.4) buffer containing 50 mM NaCl, 5 mM DTT, 100 μg μl<sup>−1</sup> bovine serum albumin, and 5% (v/v) glycerol. Polymerase catalysis was stopped by the addition of 500 mM EDTA (pH 9.0). Substrate and product DNA was separated by electrophoresis on a 20% polyacrylamide (w/v)/7 m urea gel. The products were then visualized using a phosphorimaging device and quantitated using Quantity One<sup>TM</sup> software. Results obtained under single-turnover conditions were fit to Equation 2,

\[
y = A(1 - e^{-k_{act}t})
\]

where A = product formed in first binding event, k<sub>act</sub> = rate constant defining polymerization under the conditions used for the experiment being analyzed, and t = time. Results obtained under conditions that allowed a second round of Dpo4-DNA binding and polymerase action were fit to Equation 3,

\[
y = A(1 - e^{-k_{act}t}) + k_{ss}t
\]

where k<sub>ss</sub> represents a steady-state velocity of nucleotide incorporation. To obtain an estimate of the nucleotide binding affinity for each mutant, the concentration of dNTP in the reaction mixture was varied, and pre-steady-state experiments were performed under excess enzyme conditions. The resulting rate constants, k<sub>obs</sub>, were then plotted as a function of dNTP concentration, and the data were fit to the hyperbolic expression

\[
k_{obs} = (k_{pu}[dNTP])/(dNTP) + k_{D,dNTP}
\]

using GraphPad Prism.

*LC-MS/MS Analysis of Oligonucleotide Products from Dpo4 Reactions—Dpo4 (5 μM) was preincubated with primer-template DNA (10 μM), and the reaction was initiated by the addition of dNTP (1 mM each) and MgCl<sub>2</sub> (5 mM) in a final volume of 100 μl. Dpo4 catalysis was allowed to proceed at 37 °C for 4 h in...
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As a general measure of how Dpo4 catalysis opposite 8-oxoG is affected by mutating the Arg\textsuperscript{332} residue, a time course was performed for each mutant in the presence of all four dNTPs (Fig. 1). An observed rate constant defining five incorporation events can be measured by following the appearance of the fully extended 18-mer primer and fitting the data to Equation 1, where \( n = 5 \) (Table 1). The Ala\textsuperscript{332} and Glu\textsuperscript{332} mutants were each \(-2\)–\(-6\) fold faster than any of the mutants at full-length extension opposite unmodified DNA than WT Dpo4 or the other mutants. However, WT Dpo4 was \(-2\)–\(-6\) fold faster than any of the mutants at full-length extension opposite 8-oxoG. To address the potential

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**RESULTS**

Primer Extension Past G and 8-OxoG Using All Four dNTPs—

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A 13/18-mer, $G = G$ or 8-oxoG

5' -GGG AGG ATT CNN NNN-3'  
3' -CCC CCT TCC TAA GGC ACT-5'  

18mer

FIGURE 1. Comparison of full-length extension as catalyzed by wtDpo4 and Arg$^{332}$ mutants. A, the sequence of primer-template DNA and a representative gel of full-length extension experiments. WT Dpo4 (100 nm) catalysis opposite 8-oxoG-modified DNA (100 nm) in the presence of a 1 mM mixture of all four dNTPs is shown. All experiments were performed in the presence of 1 µM unlabeled DNA (protein trap) to create true single-turnover conditions. B, formation of fully extended 18-mer product opposite unmodified DNA was quantified and plotted as a function of time for WT Dpo4 and the mutants (1). A or C added to the 3' terminus of the primer in what is assumed to be a template-independent manner (blunt-end addition). The range of misincorporation was small (11.1 ± 0.4% A incorporation for Ala$^{331}$Ala$^{332}$ and 16.0 ± 0.8% A incorporation for His$^{332}$). The trend in fidelity of 8-oxoG bypass predicted from the LC-MS/MS analysis of the oligonucleotide products was: Ala$^{331}$Ala$^{332}$ = WT Dpo4 ≈ Glu$^{332}$ (highest C incorporation) > Leu$^{332}$ > Ala$^{332}$ = His$^{332}$ (lowest C incorporation).

TABLE 1

Transient-state kinetic parameters describing full-length extension of 13/18-mer primer-template DNA

| Enzyme     | $k_{pol}$ | Template base | Product amplitude |
|------------|-----------|---------------|-------------------|
| WT         | -G-       | 85 ± 3        | 0.10 ± 0.007      |
| WT         | -8-oxoG-  | 98 ± 1        | 0.48 ± 0.010      |
| Ala$^{332}$| -G-       | 96 ± 1        | 0.21 ± 0.007      |
| Ala$^{332}$| -8-oxoG-  | 95 ± 2        | 0.18 ± 0.009      |
| Leu$^{332}$| -G-       | 90 ± 1        | 0.11 ± 0.002      |
| Leu$^{332}$| -8-oxoG-  | 92 ± 2        | 0.08 ± 0.003      |
| Glu$^{332}$| -G-       | 87 ± 2        | 0.24 ± 0.015      |
| Glu$^{332}$| -8-oxoG-  | 94 ± 1        | 0.19 ± 0.006      |
| His$^{332}$| -G-       | 89 ± 1        | 0.13 ± 0.003      |
| His$^{332}$| -8-oxoG-  | 94 ± 1        | 0.10 ± 0.003      |
| Ala$^{331}$Ala$^{332}$| -G-        | ND            | ND                |
| Ala$^{331}$Ala$^{332}$| -8-oxoG-  | ND            | ND                |

The experimental conditions were:

The data were fit to Equation 1 (Table 1).

(\begin{align*}
\text{Extended product} & = k_{pol} \times [\text{Dpo4}] \times [\text{DNA}] \\
\text{Extended product} & = K_{D,D\text{NTP}} \\
\text{Extended product} & = K_{D,d\text{CTP}} \\
\end{align*})

The kinetic parameters for each mutant were obtained in the kinetic approaches highlight the first insertion event opposite 8-oxoG, as opposed to LC-MS/MS analysis, which was conducted under conditions that allow multiple rounds of binding and dissociation.

Dpo4 Ala$^{332}$ displays several interesting characteristics. The maximum forward rate of polymerization for Ala$^{332}$ ($k_{pol}$) was 4.4- and 4.9-fold faster than observed previously with WT Dpo4 for unmodified G and 8-oxoG-modified substrates, respectively (20). The Ala$^{332}$ mutant also displayed a higher affinity (lower $K_{D,d\text{CTP}}$) for dCTP when bound to the unmodified event, but full-length extension by the double mutant did occur under conditions that allowed multiple binding events (supplemental Fig. S1). It is important to note that in the absence of any other evidence, the exact identity of the fully extended products is unknown.
fied DNA compared with the $K_{\text{d,CTP}}$ measured for Ala$^{332}$-catalyzed incorporation of dCTP opposite 8-oxoG. In the previous work with WT Dpo4, a greater affinity for dCTP was observed for 8-oxoG-modified DNA ($K_{\text{d,CTP}} = 27 \mu M$ for 8-oxoG; $K_{\text{d,CTP}} = 420 \mu M$ for G). Comparison of WT Dpo4 and Ala$^{332}$ catalytic efficiency reveals that the mutant was 23-fold more

TABLE 2
Summary of full-length extension products determined from LC-MS/MS analysis
Experiments were performed in triplicate ($n = 3 \pm \text{S.D.}$).

| Product                      | % of total product | WT Dpo4 | Ala$^{332}$ | Glu$^{332}$ | Leu$^{332}$ | His$^{332}$ | Ala$^{332}$/Ala$^{332}$ |
|------------------------------|--------------------|---------|-------------|-------------|-------------|-------------|-------------------------|
| 5'-TCGGTGA-3'                |                    | 37.0 ± 3.0 | 30.8 ± 6.0  | 0.6 ± 0.5   | 21.6 ± 2.4  | 10.3 ± 0.5  | 61.7 ± 1.9              |
| 3'-AGGCACT-5'                |                    | 33.9 ± 1.8 | 44.1 ± 5.1  | 50.0 ± 0.1  | 56.3 ± 1.6  | 60.0 ± 0.7  | 6.5 ± 0.2               |
| 5'-TCGGTGA-3'                |                    | 17.7 ± 1.0 | 9.5 ± 0.7   | 38.0 ± 0.9  | 7.8 ± 0.7   | 13.7 ± 0.1  | 23.9 ± 0.9              |
| 3'-AGGCACT-5'                |                    | 5.5 ± 0.7  | 7.2 ± 0.7   | 0.6 ± 0.1   | 3.2 ± 0.2   | 2.5 ± 0.2   | 4.6 ± 0.8               |
| 5'-TCGGTGA-3'                |                    | 5.9 ± 0.5  | 8.4 ± 1.4   | 10.9 ± 0.5  | 11.1 ± 1.2  | 13.5 ± 0.8  | 3.3 ± 0.2               |
| Total C (%)                  |                    | 88.7 ± 0.4 | 84.3 ± 1.0  | 88.5 ± 0.5  | 85.7 ± 1.2  | 84.0 ± 0.8  | 88.9 ± 0.9              |
| Total A (%)                  |                    | 11.3 ± 0.4 | 15.7 ± 1.0  | 11.5 ± 0.5  | 14.3 ± 1.2  | 16.0 ± 0.8  | 11.1 ± 0.9              |

FIGURE 2. Identification of Dpo4 mutant-catalyzed full-length extension products by LC-MS/MS. A, total ion current trace of products derived from Ala$^{332}$-catalyzed extension of 13/18-mer DNA containing 8-oxoG. B, ESI mass spectrum of the oligonucleotide peaks that elute at 3.4 min. C, total ion current trace of ion $m/z$ 1087. D, CID mass spectrum of ion $m/z$ 1087. ‘G’ denotes 8-oxoG. This product contained C inserted opposite 8-oxoG and extended in an error-free manner.
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**FIGURE 3.** Determination of k<sub>pol</sub> and K<sub>D,dNTP</sub> for Ala<sup>332</sup>-catalyzed incorporation opposite G and 8-oxoG. A, measurement of Ala<sup>332</sup>-catalyzed (200 nM) incorporation opposite G (100 nM) at varying concentrations of dCTP. B, measurement of Ala<sup>332</sup>-catalyzed (200 nM) incorporation opposite 8-oxoG (100 nM) at varying concentrations of dCTP. C, measurement of Ala<sup>332</sup>-catalyzed incorporation opposite 8-oxoG at varying concentrations of dATP. D, the observed rate of nucleotide incorporation was plotted as a function of dNTP concentration and fit to a quadratic equation to yield kinetic parameters (Table 3). Similar results were obtained for the other mutants.

| Enzyme     | Primer/template | dNTP   | k<sub>pol</sub> | K<sub>D,dNTP</sub> |
|------------|-----------------|--------|----------------|-------------------|
| WT Dpo4    | -G-             | dCTP   | 1.4<sup>a</sup> | 240<sup>b</sup>   |
|            | -8oxoG-         | dCTP   | 1.6<sup>a</sup> | 27<sup>b</sup>    |
|            | -8oxoG-         | dATP   | 4.9 ± 0.3      | 74 ± 23           |
| Ala<sup>332</sup> | -G-             | dCTP   | 4.9 ± 0.3      | 74 ± 23           |
|            | -8oxoG-         | dCTP   | 8.0 ± 0.5      | 171 ± 43          |
| Glu<sup>332</sup>  | -G-             | dCTP   | 12.2 ± 0.7     | 93 ± 43           |
|            | -8oxoG-         | dCTP   | 5.3 ± 0.8      | 742 ± 244         |
| Leu<sup>332</sup>  | -G-             | dCTP   | 5.6 ± 0.3      | 165 ± 34          |
|            | -8oxoG-         | dATP   | 0.70 ± 0.1     | 349 ± 174         |
| His<sup>332</sup>  | -G-             | dCTP   | 4.5 ± 0.6      | 520 ± 167         |
|            | -8oxoG-         | dCTP   | 2.1 ± 0.3      | 110 ± 63          |
|             | -8oxoG-         | dATP   | 0.48 ± 0.07    | 141 ± 89          |
| Ala<sup>331</sup>Ala<sup>332</sup> | -G-             | dCTP   | 4.0 ± 0.3      | 224 ± 68          |
|            | -8oxoG-         | dCTP   | 7.3 ± 0.8      | 658 ± 170         |
|            | -8oxoG-         | dATP   | 1.0 ± 0.2      | 293 ± 167         |
|            | -8oxoG-         | dATP   | 0.19 ± 0.01    | 15 ± 5            |
|            | -8oxoG-         | dATP   | 0.71 ± 0.04    | 30 ± 13           |

*<sup>a</sup> Determined previously (20).  
*<sup>b</sup> Not determined because of lack of sufficient product formation.

TABLE 3

Transient-state kinetic parameters for dCTP and dATP incorporation opposite 8-oxoG

In kinetic terms, the Ala<sup>332</sup> mutant was slightly more efficient at accurate bypass of G compared with 8-oxoG.

The Glu<sup>332</sup> mutant also exhibited slightly different kinetics from WT Dpo4. For instance, the k<sub>pol</sub> value for dCTP incorporation opposite G was 4.8-fold faster than for WT Dpo4, similar to Ala<sup>332</sup>. The k<sub>pol</sub> value for dCTP incorporation opposite 8-oxoG was 3.5-fold faster than WT Dpo4 insertion of dCTP opposite 8-oxoG. The lower K<sub>D,dCTP</sub> observed with WT Dpo4 for the 8-oxoG substrate relative to G was also apparent with the Glu<sup>332</sup> mutant, but the absolute affinity of the Glu<sup>332</sup> mutant for dCTP was diminished for both G and 8-oxoG. The catalytic efficiency of the Glu<sup>332</sup> mutant was 2.3-fold greater than WT Dpo4 for dCTP incorporation opposite G but 1.7-fold less efficient than WT Dpo4 for incorporation opposite 8-oxoG. Overall the Glu<sup>332</sup> mutant was ~5-fold more efficient at incorporating C opposite 8-oxoG compared with insertion opposite unmodified G.

The Leu<sup>332</sup> mutant can be hypothesized to serve as an intermediate between the Ala<sup>332</sup> and Glu<sup>332</sup> mutations in that the potential for hydrogen bonding between residue 332 and 8-oxoG is lost, but the steric occupancy of the Leu aliphatic side chain is retained, similar to the Glu<sup>332</sup> mutant. Pre-steady-state kinetic analysis revealed that the k<sub>pol</sub> values for the Leu<sup>332</sup> mutant incorporation opposite G and 8-oxoG were 4.1- and 1.9-fold faster than those for WT Dpo4. The lower K<sub>D,dCTP</sub> when the Leu<sup>332</sup> mutant incorporates C opposite 8-oxoG (compared with K<sub>D,dCTP</sub> for insertion opposite G) was similar to both WT Dpo4 and the Glu<sup>332</sup> mutant, but the absolute value for Leu<sup>332</sup> catalyzed bypass of 8-oxoG was increased relative to WT Dpo4. The Leu<sup>332</sup> mutant was still ~2-fold more efficient at incorporation of dCTP opposite 8-oxoG compared with G, but that difference was smaller than what is observed with WT Dpo4 and Glu<sup>332</sup>.

The His<sup>332</sup> mutant exhibited faster forward rate constants relative to WT Dpo4. The k<sub>pol</sub> values for the His<sup>332</sup> mutant incorporation opposite G and 8-oxoG were 3.6- and 4.6-fold faster than for WT Dpo4. The nucleotide binding affinity trend was opposite that of WT Dpo4, Glu<sup>332</sup>, and Leu<sup>332</sup>, with tighter dCTP binding during bypass of G. As in the case of Ala<sup>332</sup>, the kinetic analysis indicated that His<sup>332</sup> inserted dCTP opposite G with slightly greater efficiency than opposite 8-oxoG.

The Ala<sup>331</sup>Ala<sup>332</sup> double mutant had slower forward rate constants relative to WT Dpo4 for both G and 8-oxoG. The Ala<sup>331</sup>Ala<sup>332</sup> double mutant-catalyzed insertion of dCTP opposite 8-oxoG was ~4-fold faster than dCTP insertion opposite G. The measured binding affinity of dCTP was tighter than that of WT Dpo4 for unmodified DNA, but the binding affinity of dCTP opposite 8-oxoG was similar to that observed for WT Dpo4. The catalytic efficiency for dCTP incorporation was increased ~4-fold for unmodified DNA and decreased ~2-fold for 8-oxoG-modified DNA.
Transient-state Kinetic Analysis of Dpo4 Mutants and dATP Incorporation Opposite 8-OxoG—Previous steady-state analysis indicated that Dpo4 is ~90-fold more efficient at dCTP incorporation opposite 8-oxoG compared with dATP incorporation opposite the lesion (20). Previous LC-MS/MS analysis of the full-length extension products was consistent with the steady-state results (~95% C and ~5% A incorporation). Pre-steady-state analysis of WT Dpo4 and mutant-catalyzed insertion of dATP opposite 8-oxoG was performed (Fig. 3 and Table 3). A useful comparison can be made by dividing the catalytic efficiency of dCTP incorporation by the efficiency of dATP incorporation [(kcat/Km,dATP)/kcat/Km,dCTP)]. This ratio effectively measures the kinetic preference of dCTP over dATP in the concentration range of nucleotides used here (Table 4). The kinetic parameters indicate that WT Dpo4 was the most accurate enzyme tested, although Glu332 also maintains a near wild type preference for dCTP (17-fold; Table 4). The remaining mutants all exhibited decreased substrate selectivity opposite 8-oxoG, indicating some disruption to the enzymatic properties that define high-fidelity bypass of 8-oxoG. Neither the Ala332 nor the Leu332 mutant is capable of forming a hydrogen bond with 8-oxoG. The His332 residue is apparently ineffective at forming a hydrogen bond with 8-oxoG at pH 7.4 (the pK_a of the N-3 atom on the imidazole ring is presumably ~6.0), consistent with the view that hydrogen bonding between Arg332 and the O-8 atom of 8-oxoG is important for accurate and efficient bypass of the lesion. The loss of fidelity observed with Ala332 is driven by a much tighter binding of dATP relative to that observed with WT Dpo4 (Table 3), indicating that the Hoogsteen pair is better accommodated by the mutant. Likewise, the Leu332 and His332 mutants had lower K_d,dATP values than WT Dpo4 but not as low as Ala332.

Of all of the enzymes tested, only the Ala331Ala332 double mutant failed to incorporate dATP opposite 8-oxoG in the pre-steady-state experiments. Steady-state experiments revealed that the Ala331Ala332 double mutant inserted dCTP opposite 8-oxoG with an ~200-fold greater efficiency than it did dATP (supplemental Table S6). The overall steady-state efficiency for dCTP insertion opposite 8-oxoG was decreased ~12-fold relative to WT Dpo4, and the steady-state efficiency of dATP incorporation was decreased ~27-fold relative to WT Dpo4.

### Table 4

Summary of catalytic efficiencies

| Enzyme          | kcat/Km,dCTP | kcat/Km,dATP |
|-----------------|--------------|--------------|
| WT Dpo4         | 0.003 × 10^{-3} mM/s | 0.001 mM/s |
| Ala332          | 0.006 mM/s | 0.006 mM/s |
| Glu332          | 0.007 mM/s | 0.007 mM/s |
| Leu332          | 0.009 mM/s | 0.009 mM/s |
| His332          | 0.018 mM/s | 0.018 mM/s |
| Ala331Ala332    | 0.013 mM/s | <0.00× |

### FIGURE 4

Next-base extension of 8-oxoG:C or 8-oxoG:A pair. Mutant Dpo4 (200 nM) was incubated with primer-template DNA (100 nM) containing 8-oxoG template annealed to a primer containing C (○) or A (□) paired opposite the lesion. The results for dGTP incorporation were then fit to Equation 3, and the following parameters were obtained. Panel A, Ala332: C ○ at 82 ± 3 nM, kobs = 1.8 ± 0.3 s^{-1}; A (□) A at 85 ± 3 nM, kobs = 1.7 ± 0.2 s^{-1}. Panel B, Glu332: C ○ at 72 ± 1 nM, kobs = 0.8 ± 0.05 s^{-1}; A (□) A at 80 ± 3 nM, kobs = 0.9 ± 0.1 s^{-1}. Panel C, Leu332: C ○ at 74 ± 3 nM, kobs = 0.8 ± 0.1 s^{-1}; A (□) A at 79 ± 2 nM, kobs = 0.7 ± 0.06 s^{-1}. Panel D, His332: C ○ at 77 ± 2 nM, kobs = 1.0 ± 0.1 s^{-1}; A (□) A at 76 ± 3 nM, kobs = 0.9 ± 0.09 s^{-1}. Panel E, Ala331Ala332: C ○ at 9 ± 1 nM, kobs = 0.14 ± 0.3 s^{-1}; A (□) A at 60 ± 8 nM, kobs = 0.031 ± 0.006 s^{-1}. The concentration of dGTP in all 10 experiments was 1 mM.
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### TABLE 5
Crystal data and refinement parameters for the ternary (protein-DNA-dGTP) complexes of Dpo4 mutants

| Parameter | R332E(8-oxoG:A) | R332E(8-oxoG:C) | R332A(8-oxoG:A) | R332A(8-oxoG:C) |
|-----------|-----------------|-----------------|-----------------|-----------------|
| X-ray source | APS (DND-CAT) | APS (DND-CAT) | APS (DND-CAT) | APS (IMCA-CAT) |
| Detector | MARCCD | MARCCD | MARCCD | Quantum CCD |
| Wavelength (Å) | 1.00 | 1.00 | 1.00 | 1.00 |
| Temperature (K) | 110 | 110 | 110 | 110 |
| No. of crystals | 1 | 1 | 1 | 1 |
| Space group | P2<sub>1</sub>,2 | P2<sub>1</sub>,2 | P2<sub>1</sub>,2 | P2<sub>1</sub>,2 |
| Unit cell (a, b, c; Å) | 96.77,103.72,52.91 | 95.77,103.72,53.03 | 95.13,104.12,52.94 | 94.72,104.19,52.72 |
| Resolution range (Å) | 32.258–2.092 | 29.323–2.20 | 29.260–2.70 | 46.078–2.9 |
| No. of measurements | 105,683 (6,277) | 175,452 (17,731) | 75,984 (8,047) | 50,447 (3,377) |
| No. of unique reflections | 29,759 (2,221) | 27,480 (2,697) | 14,782 (1,532) | 11,732 (965) |
| Completeness (%) | 92.3 (70.2) | 99.1 (98.6) | 97.9 (98.9) | 98.3 (89.0) |
| Redundancy | 3.45 (2.8) | 6.38 (6.57) | 5.14 (5.25) | 4.3 (3.5) |
| Signal to noise (I/σ(I)) | 4.71 (43.66) | 5.3 (43.8) | 8.8 (55.9) | 13.7 (60.7) |
| Solvent content (%) | 54.7 | 55.3 | 54.2 | 55.5 |
| Mean isotropic (Å<sup>2</sup>) | 46.12 | 40.32 | 54.31 | 49.42 |
| R<sub>f</sub> (%) | 24.87 | 22.76 | 21.56 | 23.21 |
| R<sub>merge</sub> (%) | 27.31 | 25.71 | 26.96 | 26.54 |
| Wilson plot (Å<sup>2</sup>) | 12.433 (1.5) | 23.61 (4.41) | 14.25 (7.11) | 8.72 (1.80) |

**Relevant Excerpts**

**Mutant Dpo4-catalyzed Extension beyond 8-OxoG Paired with C or A**—The next-base extension of 8-oxoG:C and 8-oxoG:A pairs was measured by performing pre-steady-state experiments at a high concentration of the incoming dGTP (Fig. 4). All of the single mutants extended the 8-oxoG:C and 8-oxoG:A pairs in a similar manner, suggesting that next-base extension was relatively unaffected by the identity of the pair being extended. The rate of extension of the 8-oxoG:C pair was decreased for all four mutants relative to the 8-oxoG:A pair, but the rate and the amplitude of product formation were both decreased. The double mutant extended the A:8-oxoG pair, but the rate was very slow.

**R332A Mutant-DNA Complex Structures**—The structures of the Ala<sup>332</sup> mutant were determined in complex with a DNA duplex (18-mer template and 14-mer primer) containing either an 8-oxoG:A or an 8-oxoG:C base pair and an incoming dGTP at the active site (termed R332A(8-oxoG:A) and R332A(8-oxoG:C), respectively; Table 5 and supplemental Fig. S31). Both the R332A(8-oxoG:A) and the R332A(8-oxoG:C) structure represent type I complexes (11). The template C located 5′ to 8-oxoG is accommodated inside the active site of both the R332A(8-oxoG:A) and the R332A(8-oxoG:C) structures and pairs with dGTP at the replication site (Fig. 5, A and B). The 8-oxoG pairs with the 3′-terminal base of the primer at the post-insertion (−1) site. The short side chain of Ala<sup>332</sup> allows considerable space for accommodating 8-oxoG:C or 8-oxoG:A pairs (Fig. 6, A and B). 8-oxoG is in a syn conformation in the R332A(8-oxoG:A) complex and forms a Hoogsteen pair with A. In this complex the O-8 atom forms a water-mediated hydrogen bond with Tyr<sup>12</sup> (Fig. 5A). The R332A(8-oxoG:C) complex shows the 8-oxoG residue in an anti conformation and forms a Watson-Crick paired with C. Lys<sup>78</sup> forms a water-mediated hydrogen bond to the N-2 of 8-oxoG (Fig. 5B). Such water-mediated hydrogen bonding interactions involving Tyr<sup>12</sup> or Lys<sup>78</sup> were
not observed in the WT Dpo4 complexes (Fig. 5, E and F) (20). There, the 8-oxoG pairs with the dNTP at the replicative position. The Tyr12 and Lys78 side chains are directed toward the base pair in the /H11002 position both in the WT Dpo4 and mutant structures (Fig. 5). Therefore, the presence of the water-mediated hydrogen bonds observed in case of the R332A mutant structures suggests that these residues may play a role in translocation of the primer-template duplex and may not be critical with regard to the insertion event.

**R332E Mutant-DNA Complex Structures**—The Glu332 mutant protein was complexed with an 18-mer template-14-mer primer DNA duplex containing either 8-oxoG:A or 8-oxoG:C pair at the active site and an incoming dGTP (termed R332E(8-oxoG:A) and R332E(8-oxoG:C), respectively; supplemental Fig. S31). As with the complexes of Dpo4 alanine mutants above, the template C located 5′ to 8-oxoG pairs with dGTP and 8-oxoG pairs with the 3′-terminal base of the primer at the −1 site. 8-oxoG pairs in the Hoogsteen mode with A in the R332E(8-oxoG:A) structure (Fig. 5C). Here, Glu332 engages in a direct but relatively long hydrogen bond (3.46 Å) as well as a water-mediated interaction with the exocyclic amino group N-2 of 8-oxoG. The R332E(8-oxoG:C) structure reveals a 8-oxoG:C pair in a Watson-Crick configuration with the O-8 oxygen linked to Glu332 via two water molecules (Fig. 5D). The side chain of Glu332 fills most of the space near the 8-oxoG base (Fig. 6, C and D). The available space is thus clearly reduced compared with the Ala332 mutant structures but still is more open than in the WT Dpo4 structures. The presence of the long side chain of Arg332 in WT Dpo4 (Fig. 6, E and F) may influence the choice of the inserted nucleotide. Steric hindrance and resulting repulsive interactions may take place in the case of the wild type 8-oxoG:dATP complex, leading to a destabilization of the Hoogsteen pair between 8-oxoG and dATP. Conversely, formation of a hydrogen bond between Arg332 and O-8 can be expected to stabilize the Watson-Crick 8-oxoG: dCTP pair in the wild type 8-oxoG: dCTP complex (20), thus providing a rationalization of the preferred incorporation of C opposite 8-oxoG by Dpo4.

**DISCUSSION**

Mechanisms of cellular dysfunction, including carcinogenesis and aging, are caused, in part, by covalent modification of DNA (13, 32). 8-oxoG is an important lesion to consider because it is widely prevalent and known to be mutagenic. Many of the DNA polymerases studied in vitro incorporate a large fraction of A opposite 8-oxoG relative to C (33–39). Some polymerases, such as yeast pol(40, 41) and RB69 (42), preferentially insert dCTP opposite 8-oxoG, but the ability of Dpo4 to bypass 8-oxoG in a manner that is not only accurate but also more efficient than catalysis opposite unmodified DNA makes it unique among the DNA polymerases studied to date (20). Previous work suggested that a hydrogen bond between Arg332 and the O-8 atom of 8-oxoG facilitates the increased efficiency of Dpo4 catalysis (20, 21). The role of Arg332 in facilitating bypass efficiency was examined by studying the structure and mechanism of four mutant enzymes.

There are two major points to consider when discussing Dpo4-catalyzed bypass of 8-oxoG. First, the accuracy (or fidelity) of the reaction is high. The role of Arg332 may be to help stabilize the anti conformation of the purine ring system of 8-oxoG, which would favor the 8-oxoG:C Watson-Crick-like pair over the 8-oxoG:A Hoogsteen pair. The second major point to consider is the matter of enzyme efficiency. Does Arg332 play a predominant role in increasing the efficiency of
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8-oxoG bypass, or is efficiency determined by several interactions between Dpo4 and the template DNA?

On the issue of fidelity, changing the identity of the Arg$^{332}$ residue does not result in obvious changes to Dpo4 catalysis opposite unmodified DNA (Fig. 1 and LC-MS data not shown). Relatively subtle changes in fidelity were observed in LC-MS/MS analysis of the full-length extension products for 8-oxoG-modified DNA (Table 2). The Glu$^{332}$ mutant exhibits fidelity that is very similar to WT Dpo4 (~11% A incorporation). With the exception of the Ala$^{331}$Ala$^{332}$ double mutant, the other mutants incorporated more A, indicating that some interaction was lost that moved the equilibrium between accurate and mutagenic bypass of 8-oxoG toward incorporation of A. The kinetic parameters are consistent with the LC-MS/MS results. The predicted trend in fidelity of 8-oxoG bypass is as follows: WT Dpo4 = Ala$^{331}$Ala$^{332}$ (highest C incorporation) > Glu$^{332}$ > Leu$^{332}$ > Ala$^{332}$ = His$^{332}$ (lowest C incorporation). One interesting comparison here is between the apparent preference, as determined by kinetic efficiency, and the products identified by LC-MS/MS. The kinetic parameters indicate that WT Dpo4 and the Glu$^{332}$ would insert ~5–6% A compared with ~15% A for Leu$^{332}$ and ~27% A for the Ala$^{332}$ and His$^{332}$ double mutant in complex with the C:8-oxoG pair, but the little finger domain is disordered, which makes it difficult to discern why the double mutant retains such high fidelity opposite 8-oxoG (data not shown). On the other hand, the full-length extension results with the Ala$^{331}$Ala$^{332}$ double mutant clearly show an effect upon Dpo4 processivity. Indeed, all of the enzymes tested here are slower at next-base extension of the 8-oxoG:C pair, indicating the importance of positively charged residues in the little finger domain during the Dpo4 translocation step. Such a view is consistent with previous structural work showing that Arg$^{332}$ maintained contact with 8-oxoG even when the template base is shifted into the post-insertion site of a type II structure (20).

The second major point to consider is the efficiency of Dpo4-catalyzed bypass of 8-oxoG. In this regard, the Glu$^{332}$ mutant is most similar to WT Dpo4. The Glu$^{332}$ mutant is 5-fold more efficient at inserting dCTP opposite 8-oxoG compared with G. The Leu$^{332}$ mutant is 2-fold more efficient inserting dCTP opposite 8-oxoG compared with G, but the Ala$^{332}$ and His$^{332}$ mutants are slightly more efficient at inserting dCTP opposite G. With the exception of Ala$^{332}$, which inserts dCTP opposite G with ~20-fold greater efficiency than WT Dpo4 (Table 4), the
gap between efficiency of G and 8-oxoG bypass is caused primarily by a decrease in bypass efficiency opposite 8-oxoG (Table 4). As with the issue of fidelity, the differences in efficiency are not large, but the Glu332 mutant is the only one of these mutants capable of effectively forming a hydrogen bond with 8-oxoG, and it is the most similar to WT Dpo4 in kinetic terms.

The fact that the Glu332 mutant is similar to WT Dpo4 contradicted our initial hypothesis that the negatively charged side chain of Glu332 would move 8-oxoG into the Hoogsteen mode. The crystal structures reveal how the similarities between WT Dpo4 and Glu332 are maintained. A water-mediated hydrogen bond is formed between the carboxylic acid moiety of Glu332 and the O-8 atom of 8-oxoG (Fig. 5C). The Glu332 mutant also forms a hydrogen bond with the N-2 exocyclic amino group of 8-oxoG when the 8-oxoG:A Hoogsteen pair is formed (Fig. 5D). No such interaction is observed in the structure of WT Dpo4 and the 8-oxoG:A Hoogsteen pair (20), but it is unclear what effect a hydrogen bond between Glu332 and the N-2 amino group of 8-oxoG has upon dATP insertion, as the efficiency is not increased relative to WT Dpo4 (Table 4).

An interesting difference between WT Dpo4 and the Ala332 mutant is the increased proclivity to insert dATP, which reduces the fidelity of the Ala332 mutant. The Ala332 mutant crystal structures (Fig. 6, A and B) reveal an empty space in the region normally occupied by Arg332 in WT Dpo4. The Arg331 residue maintains contact with the template DNA to the 5’-side of the 8-oxoG lesion and does not change conformation to replace Arg332. The increased ability of the Ala332 mutant to insert dATP opposite 8-oxoG may be related to the syn/anti equilibrium in the Dpo4 active site. A larger space could be more accommodating to the 8-oxoG:A Hoogsteen pair, as evidenced by the relatively low $K_{dATP}$ measured for Ala332 insertion of dATP opposite 8-oxoG (Table 3).

In comparing the mechanism for Dpo4-catalyzed bypass of 8-oxoG with other DNA polymerases, an important similarity to bacteriophage pol T7 is observed. The crystal structure of pol T7 in ternary complex with a 8-oxoG:ddCTP pair revealed that Lys536 is in position to form a hydrogen bond with the O-8 atom of 8-oxoG (33). The side chain of Lys536 moves 3 Å relative to the position observed in a superimposed structure of pol T7 bound to unmodified DNA. The stabilization of 8-oxoG by a hydrogen bond bears obvious resemblance to Dpo4, but it is estimated that pol T7 inserts A opposite 8-oxoG in ~30% of incorporation events, even when exonuclease activity is present (33). Insertion of dCTP opposite 8-oxoG by pol T7 is inhibited ~180-fold relative to insertion of dCTP opposite G (comparing pre-steady-state data) (36). Both the level of dATP incorporation and the catalytic inhibition of pol T7 is in direct contrast to what has been observed with Dpo4. The stabilization of 8-oxoG by Lys536 in the pol T7 structure is apparently not substantial enough to overcome other factors, i.e. a geometrically intolerant active site and kinking of the template DNA backbone, to facilitate high efficiency and high fidelity during T7-catalyzed bypass of 8-oxoG.

A sequence alignment of Dpo4 with Saccharomyces cerevisiae pol η based on secondary structure predicts that a histidine residue should be found in the region occupied by Arg332 (11). However, a structure-based alignment of the little finger domain from the two proteins suggests that Arg332 is replaced by a lysine (Lys498) in S. cerevisiae pol η (Fig. 7). The results presented here may be consistent with our structure-based alignment, because the His332 mutant has the lowest fidelity of all of the mutants tested here, whereas yeast pol η is known to bypass 8-oxoG with relatively high fidelity (40). If our structure-based alignment is correct then the electrostatic interaction between the little finger domain and 8-oxoG may also be important for high-fidelity translesion synthesis opposite 8-oxoG by pol η.
The hydrogen bond between Arg$^{332}$ and 8-oxoG is probably not the only factor affecting Dpo4 fidelity/efficiency during bypass of the lesion. There are, in fact, several other charged residues in the little finger that make important interactions with the phosphate backbone of DNA (including Arg$^{242}$, Arg$^{247}$, Lys$^{275}$, Arg$^{298}$, Arg$^{331}$, and Arg$^{336}$). The array of positively charged residues may help guide the anti conformation of 8-oxoG into and out of the active site of Dpo4, consistent with interactions predicted by computational studies (43). Changing the nature of one of these residues does not appear to dramatically alter enzyme efficiency, even if it is the residue that contacts 8-oxoG during insertion opposite the lesion. Another possible reason for the small changes in fidelity and efficiency observed with the mutants emerges when one superimposes WT Dpo4 structure and either the Glu$^{332}$ or Ala$^{332}$ mutant structures. In the all of the structures, Ala$^{42}$ engages in a C(H)$_3$⋯π interaction with the templating base that helps to define the “roof” of the Dpo4 active site. When 8-oxoG is in the anti conformation, ready to pair with the incoming dCTP in a Watson-Crick mode, the “stacking” interaction with Ala$^{42}$ is more favorable than when 8-oxoG assumes the syn conformation. In the syn orientation the six-member ring of 8-oxoG extends out into the major groove and does not interact with Ala$^{42}$. Interestingly, the active site of T7$^\text{37}$ has a glycine residue (Gly$^{527}$; Protein Data Bank accession code 1TK0) in place of alanine. In pol T7$^\text{37}$ the contact between Gly$^{527}$ and the template base is less intimate, with only a single hydrogen atom directed toward the nucleobase. It is possible that the interaction with Ala$^{42}$ in WT Dpo4 favors the anti conformation of 8-oxoG and in combination with the Arg$^{332}$ hydrogen bond in WT Dpo4 effectively seals the preference for incorporation of dCTP. In the case of the Ala$^{332}$ mutant, the hydrogen bond at position 332 is absent, but the stacking interaction between 8-oxoG and Ala$^{332}$ may still promote a relatively high-fidelity mechanism of bypass. Of the mutants tested here, Glu$^{332}$ maintains catalytic properties during bypass of 8-oxoG that are most similar to WT Dpo4. A water-mediated hydrogen bond between Glu$^{332}$ and the O-8 atom of 8-oxoG provides further evidence that Dpo4 does use a hydrogen bond with the O-8 atom of 8-oxoG as an electrostatic “handle” that participates in increasing the fidelity of bypass and, to some extent, as a means of increasing catalytic efficiency.

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