2016

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Importance of Ile716 toward the mutagenic potential of 8-oxo-2’-deoxyguanosine with polymerase I from Bacillus Stearothermophilus

by

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Honors Thesis

in

Program in Biochemistry and Molecular Biology
University of Richmond
Richmond, VA

4/22/16

Advisor: Dr. Michelle Hamm
This thesis has been accepted as part of the honors requirements in the Program in Biochemistry and Molecular Biology.

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4/21/16
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Abstract:

8-oxo-2’-deoxyguanosine (OdG) is a promutagenic DNA lesion that arises from exposure of 2’-deoxyguanosine (dG) to reactive oxygen species (ROS) like peroxides, hydroxy radicals, and superoxides. ROS are produced by environmental carcinogens, exposure to radiation, and even during cellular respiration. OdG lesions are very common in mammalian cells and can base pair to both 2’-deoxycytidine (dC) and 2’-deoxyadenosine (dA), the latter of which can result in dG → thymidine transversions. These mutations are believed to be responsible for the link between OdG and aging, and diseases like cancer, lupus, and arthritis. To gain insight into the preference for dCTP or dATP incorporation opposite OdG, and thereby the mutagenic potential of OdG, the large fragment of the A-family polymerase I from the bacterium Bacillus Stearothermophilus (BF) was investigated. Previous studies have suggested that active site clashing between Ile716 and either the C8-position of OdG during dCTP incorporation, or the C2-position of OdG during dATP incorporation can heavily influence the mutagenic potential of OdG. To further test the importance of these clashing interactions, the activities of dCTP and dATP incorporation opposite OdG and seven of its analogues were compared between wild type BF, Ile716Met BF, and Ile716Ala BF.
**Introduction:**

Reactive oxygen species (ROS) are produced by a variety of metabolic pathways and play an important role in cellular function.\(^1\) Some examples include peroxides (R₂O₂), hydroxy radicals (•OH), and superoxide ions (O₂•⁻). Higher levels of ROS can result under conditions of increased stress, cellular respiration, or by environmental carcinogens and exposure to radiation.\(^2\) This can cause damage to cellular components and have a serious impact on normal cell function. 8-oxo-2’-deoxyguanosine (OdG) is a promutaginic DNA lesion that arises from exposure of 2’-deoxyguanosine (dG) to ROS\(^3\) (Figure 1). OdG lesions are incredibly common in mammalian cells, occurring between 1,000 and 10,000 times each day.\(^4\) OdG is considered a promutagen as it can base pair to both 2’-deoxycytidine (dC) and 2’-deoxyadenosine (dA) during replication, the latter of which can result in dG → thymidine transversions. These mutations are believed to be responsible for the link between OdG and aging, and diseases like cancer, lupus, and arthritis.\(^5\)

OdG can be found in different tautomeric states, yet predominately exists in the 6,8-diketo tautomer at physiological pH.\(^6,7\) In this structure, OdG and dG exhibit different hydrogen bonding character, due to structural dissimilarities at the N7- and C8-positions within the imidazole ring. OdG possesses an N7-hydrogen and C8-oxygen, which act as a hydrogen bond donor and acceptor, respectively. In contrast, dG has no hydrogen

![Diagram of OdG formation and tautomeric states](image)

*Figure 1: OdG is formed from exposure of dG to ROS.\(^4\) While dG prefers an anti-conformation, OdG prefers a syn conformation.*
bonding ability at the C8-position and only possesses hydrogen bond accepting ability at the N7-position. Additionally, like other nucleosides that contain a large atom at the C8-position, OdG mononucleosides prefer the syn-conformation around the glycosidic bond, in comparison to dG, which prefers the anti-conformation (Figure 1). OdG uses the anti-conformation when base pairing with dC to form a base pair that is structurally similar to a dG:dC base pair. Alternatively, OdG uses the syn-conformation when base pairing with dA to form a structure that is analogous to a dT:dA base pair (Figure 2). Melting studies have shown significant decreases in the stability of OdG:dC base pairs in comparison to dG:dC pairs, but significant increases in the stability of OdG:dA base pairs in comparison to dG:dA base pairs. These changes lead OdG:dC base pairs to be only slightly more stable than OdG:dA base pairs, which helps explain the mutagenic potential of this DNA lesion.
In addition to the thermodynamic character of OdG:dX (dC or dA) base pairs, research suggests that polymerases themselves may also influence the mutagenic potential of OdG. Polymerases may preferentially bind a template OdG in either the anti- or syn-conformation, leading to a preference for dCTP or dATP incorporation, respectively (Figure 3 and Table 1). Polymerases that preferentially incorporate dATP opposite OdG increase the mutagenic potential of this lesion, while polymerases that preferentially incorporate dCTP opposite OdG decrease the mutagenic potential.

While most polymerases exhibit a similar overall structure that resemble the shape of a right hand with “thumb,” “palm,” and “finger” domains, sequence homology, structure, and activity classify polymerases into seven different families, (A, B, C, D, X, Y, and reverse transcriptase) which are often categorized into two groups, replicative and translesion synthesis polymerases. Polymerases in families A, B, C, and D typically have a replicative role, with tight active sites that fit DNA and dNTP substrates. A-family polymerases, which includes the large fragment of Pol I from both *Escherichia coli*, (Klenow Fragment; KF-exo) and *Bacillus stearothermophilus* (BF), are well studied and have rigid active sites that conform to cognate base pair shape and are sensitive to perturbations in both the major and minor groove. B-family polymerases are the most abundant polymerases, characterized by high fidelity replicative activity. For example,

| Table 1 |
|-------------------------------|-----------------|-------|
| Polymerase | dCTP/dATP incorporation ratio opposite OdG | Family |
| Dpo4 | 91 | Y |
| RB69 | 20 | B |
| DNA pol η | 20 | Y |
| KF-exo | $7^{14}$ and $4^{15}$ | A |
| DNA pol β | 2 | X |
| BF | 0.11 | A |
| DNA pol κ | 0.007 | Y |
| DNA pol α | 0.005 | B |
DNA polymerase from bacteriophage RB69 and DNA pol α, which are both B-family polymerases, play an important role in DNA replication initiation. Polymerases in families X and Y are typically active during DNA repair or lesion bypass and have looser, more tolerant active sites. Dpo4, DNA pol η, and DNA pol κ are all Y-family polymerases, lack proofreading activity, and are known for their ability to replicate past large, damaged DNA bases. While mainly involved in translation synthesis, X-family polymerases typically have intrinsic fidelity that is comparable to many polymerases involved in DNA replication. For example, DNA pol β, a human polymerase involved in base excision repair, exhibits high fidelity, which is comparable to DNA polymerase I. The structural and biochemical variations across polymerase families result in varying preferences for dCTP or dATP incorporation and consequential mutagenicity differences between families (Table 1).

Though differences between polymerase families are unsurprising, it is important to note that dCTP/dATP incorporation ratios vary even within polymerase families. This can be seen within Y-family polymerases, as DNA pol η and Dpo4 preferentially incorporate dCTP opposite OdG, while Dpol κ preferentially incorporates dATP opposite OdG (Table 1). Additionally, A-Family Polymerases KF-exo and BF also exhibit differences in incorporation preferences. KF-exo preferentially incorporates dCTP opposite OdG with approximately 4-fold higher efficiency, while BF exhibits a 9-fold preference for dATP incorporation (Table 1). In order to understand the exact properties that determine these incorporation preferences and, consequently, the mutagenic potential of OdG, we chose to further investigate the incorporation preferences of BF.
BF was chosen for this study as it lacks a proofreading exonuclease, preferentially inserts dATP opposite OdG, and several BF crystal structures are known. BF Polymerase I contains five identified regions that contribute to high-fidelity DNA replication: the insertion, catalytic, pre-insertion, and post-insertion sites, as well as the DNA binding duplex (Figure 4). Previous research has shown that BF transitions from a closed to an open state through a bent O helix intermediate conformation. It is hypothesized that these conformational changes allow for selective incorporation of dNTPs. Furthermore, it is proposed that specific residues play a role in the dynamic structure of the enzyme, thus influencing the dCTP/dATP incorporation preference opposite OdG. The study herein looks specifically at the importance of one key major groove residue, Ile716, in determining incorporation preference and consequently, influencing the mutagenic potential of OdG.

Previous research has suggested that clashing between the branched Ile716 residue and the C8-position or C2-position of OdG during dCTP or dATP incorporation,
respectively, might influence the incorporation preference opposite OdG. To gain further insight on these clashing interactions and the importance of Ile716, dCTP and dATP incorporation activities opposite OdG and seven of its analogues were compared between wild type (WT) BF and two major groove mutants, Ile716Met BF and Ile716Ala BF. These residues were chosen in order to assess clashing differences between branched chain isoleucine, linear chain methionine, and short chain alanine residues. Understanding the differences in dATP and dCTP incorporation between these enzymes offers further information on the dynamic active site of BF and its role in the mutagenic potential of OdG.

The OdG analogues used in this study varied at the C8- and C2-positions and included: i) 2’-deoxyguanosine (dG) and 2’-deoxyinosine (dI), which both lack an N7-hydrogen and a large C8-substituent, but differ in that dG possesses a C2-exocyclic amine that is not present in dI, ii) OdG and SdG, which have an N7-hydrogen, and an oxygen and sulfur atom at the C8-position, respectively, yet differ from OdI and SdI with the presence or absence of the C2-amine, iii) CldG and BrdG, which have C8-chlorine and bromine atoms, respectively, but lack the N7-hydrogen that is necessary for pairing with dA, and iv) CdG, which has the same structure as OdG, but does not have an oxygen atom at the C8 position (Figure 5).
order to assess the importance of the C8-oxygen when incorporating dCTP, the efficiencies opposite dG, ClpG, BrdG and SdG were compared. The role of the C8-oxygen during dATP incorporation was investigated by comparing the activity opposite CdG, OdG, and SdG, as well as OdI and SdI. Finally, activity differences opposite OdI, and SdI as compared to OdG and SdG, respectively, offered insight on the importance of the C2-exocyclic amine during dATP incorporation. Studying the influence of steric bulk and electrostatics at the C8- and C2-positions using these analogues is essential to understanding the mutagenic potential of OdG.

**Experimental**

**Production of Wild Type and Mutant enzymes**

All three BF enzymes (WT, Ile716Met and Ile716Ala) were synthesized and purified by the Eugene Wu lab at the University of Richmond.

**Incorporation of dNTP opposite OdG and other analogues using WT BF and Mutants**

Reaction solutions were prepared containing Tris-HCl, MgCl₂, DTT, BSA, template, and ³²P radiolabeled primer. These solution were heated at 90° C for three minutes before cooling to room temperature for thirty minutes. BF was then added to each solution, which resulted in the following final concentrations: 10 mM Tris-HCl, 1 mM MgCl₂, 0.2 mM DTT, 0.5 µg/mL BSA, 0.4 µM template, and 0.4 µM ³²P-radiolabeled primer. All reaction tubes were incubated at 37° C for five minutes, before adding 5 µL of the enzyme containing solution to 5 µL of a 2X dNTP solution, which
contained 20 mM NaCl and the appropriate dATP or dCTP concentration. The final concentration for WT BF experiments was 0.2 nM for all reactions, except SdG:dATP incorporation experiments, in which 2 nM enzyme concentration was used. For Ile716Ala BF and Ile716Met, the final enzyme concentrations were 1 nM and 0.1 nM, respectively. Reactions were stopped with 20 µL of a STOP solution (95% formamide, 20 mM EDTA, 0.0025 % bromophenol blue, and 0.0025 % xylene cyanol) so that reactions were ≤ 20 % complete. The incorporation reaction scheme can be seen in Figure 6.

![Reaction Scheme- Incorporation Experiment](image)

*Figure 6. Incorporation of a dNTP into a 10 nucleotide long primer opposite dG, OdG or an analogue within a 15 nucleotide long template.*

**Data Analysis**

Products and starting reactants in solution were separated with 20% denaturing PAGE. The product primer strand did not run as far as the reactant primer strand due to its one nucleotide longer-length. Gels were dried on a Bio-Rad Gel Dryer and exposed to an Amersham phosphor screen overnight. Gels were imaged with an Amersham Storm 860 Phosphorimager and the concentration of product and reactant bands were quantified using the ImageQuant version 5.0. From this data, repeated in triplicate, Michealis Menten curves were generated with Kaleidagraph. \( V_{max} \) and \( K_m \) were calculated for each experiment to determine enzyme incorporation efficiency.
Results and Discussion

Influence of the C8 and C2 positions on the mutagenic potential of OdG and its analogues using Wild Type BF

dCTP incorporation opposite OdG and its analogues

Steady state kinetic experiments were used to determine dCTP and dATP incorporation efficiencies opposite OdG and its analogues with WT BF. Incorporation of dATP opposite dG, dI, CldG and BrdG was not measured as these analogues lack the N7-hydrogen necessary for the formation of a base pair to dA. The parameters of dCTP and dATP incorporation with WT BF can be seen in Table 2.

<table>
<thead>
<tr>
<th>X:dNTP</th>
<th>Vmax (% min⁻¹)</th>
<th>Km dNTP (μM)</th>
<th>Vmax/Km (% min⁻¹ μM⁻¹)</th>
<th>dCTP/dATPb</th>
</tr>
</thead>
<tbody>
<tr>
<td>dG:dCTP</td>
<td>23 ± 5</td>
<td>0.43 ± 0.09</td>
<td>54 ± 17</td>
<td></td>
</tr>
<tr>
<td>CldG:dCTP</td>
<td>66 ± 10</td>
<td>490 ± 25</td>
<td>0.14 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>BrdG:dCTP</td>
<td>32 ± 9</td>
<td>620 ± 160</td>
<td>0.052 ± 0.019</td>
<td></td>
</tr>
<tr>
<td>CdG:dCTP</td>
<td>86 ± 18</td>
<td>69 ± 10</td>
<td>1.3 ± 0.3</td>
<td>2.8</td>
</tr>
<tr>
<td>CdG:dATP</td>
<td>73 ± 21</td>
<td>170 ± 20</td>
<td>0.44 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>OdG:dCTP</td>
<td>6.3 ± 1.4</td>
<td>370 ± 20</td>
<td>0.044 ± 0.004</td>
<td>0.29</td>
</tr>
<tr>
<td>OdG:dATP</td>
<td>7.6 ± 2.1</td>
<td>77 ± 26</td>
<td>0.15 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>SdG:dCTP</td>
<td>2.9</td>
<td>260</td>
<td>0.011</td>
<td>0.39</td>
</tr>
<tr>
<td>SdG:dATP</td>
<td>1.0</td>
<td>36</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>OdI:dCTP</td>
<td>52 ± 10</td>
<td>600 ± 120</td>
<td>0.086 ± 0.024</td>
<td>0.19</td>
</tr>
<tr>
<td>OdI:dATP</td>
<td>97 ± 14</td>
<td>220 ± 30</td>
<td>0.45 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>SdI:dCTP</td>
<td>1.8 ± 0.2</td>
<td>210 ± 20</td>
<td>0.0086 ± 0.0013</td>
<td>0.74</td>
</tr>
<tr>
<td>SdI:dATP</td>
<td>4.2 ± 0.3</td>
<td>360 ± 70</td>
<td>0.012 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>dEdCTP</td>
<td>2.7 ± 0.9</td>
<td>0.060 ± 0.010</td>
<td>48 ± 19</td>
<td></td>
</tr>
</tbody>
</table>

a: Vmax and Km values are reported as the average ± sd.
b: Incorporation preference = (Vmax/Km)₅CTX/ (Vmax/Km)₅CATP.
Comparing incorporation efficiencies across the varying analogues offers insight onto the importance of the C2- and C8-position during dCTP incorporation. The data exhibits comparable incorporation efficiency opposite dG and dI, suggesting that the presence of the C2-amine group and a third hydrogen bond to dC does not impact overall dCTP incorporation efficiency.

CldG and BrdG, which each possess a large atom at the C8-position, direct dCTP incorporation with 400- and 1000-fold decreases in dCTP incorporation efficiency as compared to dG (Table 2 and Figure 7). A similar trend is also seen when comparing dCTP incorporations opposite CdG, OdG, and SdG, as well as opposite dI, OdI, and SdI. This trend—a decrease in dCTP incorporation efficiency as the size of the C8-atom increases—suggests that WT BF is sensitive to steric interference at the C8-position. The crystal structure of BF seen in Figure 8 exhibits the position of OdG in the active site of WT BF during the dCTP incorporation. In the required anti-orientation, the C8-atom is within close proximity to Ile716 in the major groove. Therefore, the decrease in incorporation efficiency with increasing C8-steric bulk is likely due to clashing with this isoleucine residue.
dATP incorporation opposite OdG and its analogues

To assess the importance of both the C8-atom and the C2-amine group during dATP incorporation, the efficiencies of these reactions opposite the various analogues were also compared. Incorporations opposite OdG and SdG exhibited 3- and 15-fold reductions, respectively, in comparison to opposite CdG (Table 2). This is likely due to the sterically larger atoms at the C8-position in OdG and SdG. A similar trend was seen in the dI background where dATP incorporation opposite OdI was 38-fold more efficient than opposite SdI. OdG adopts the syn-conformation during dATP incorporation; thus the C8-atom would be positioned near Tyr714 in the minor groove (Figure 8). This added steric bulk and clashing between the oxygen or sulfur atom in OdX (OdG or OdI) and SdX (SdG and SdI) with this tyrosine residue are likely responsible for the decreased dATP incorporation efficiency observed with increasing atom size off C8.

Previous dATP incorporation results suggest an increase in incorporation efficiency when the C2-amine is removed. Our results are consistent; dATP incorporation
opposite OdG is 3-fold less efficient than opposite OdI. During dATP incorporation, OdG is in the syn-conformation and the C2-amine group is near Ile716 in the active site of BF, as depicted in Figure 7. Similar to the C8-position during dCTP incorporation, additional steric bulk at the C2-position likely clashes with the isoleucine residue, decreasing dATP incorporation efficiency. This trend is not seen when comparing incorporation efficiency between SdG and SdI, however, these results are just preliminary and need to be repeated.

*dCTP/dATP incorporation preference opposite OdG and its analogues*

In order to determine how steric bulk at the C8- and C2-positions confers incorporation preference, dCTP/dATP incorporation efficiencies opposite CdG, OdG, and SdG were compared. Previous research with BF indicated that analogues with a bulky atom at the C8-position (SdX and OdX) preferentially direct dATP incorporation, whereas those with small atoms at the C8-position (CdG) preferentially incorporate dCTP. Similar results were found in this study with WT BF. CdG directed dCTP incorporation with approximately 3-fold greater efficiency when compared to dATP, while OdG exhibited approximately 3-fold greater preference for dATP incorporation. Thus far, results with SdG are not consistent, but again it is important to note that the SdG and SdI results are preliminary. Members of the Hamm Lab are currently further investigating how SdG and SdI directs dATP and dCTP incorporation. Nonetheless, the results with CdG and OdG indicate that Ile716 is likely important in determining incorporation preferences, in part due to its clashing with the C8-atom during dCTP incorporation.
Comparison of incorporation efficiency opposite OdG and its analogues using WT BF, Ile716Ala BF, and Ile716Met BF

In addition to the WT BF incorporation experiments, we also looked at dNTP incorporation efficiencies with two mutant enzymes. In the first mutant polymerase, Ile716Met BF, the branched isoleucine was replaced by a straight chain methionine. Previous research has shown that KF, which contains Met768 at the site corresponding to Ile716 in BF, exhibits preferential dCTP incorporation opposite OdG. In the second mutant enzyme, Ile716Ala BF, the isoleucine residue was replaced by an alanine residue, which has a short methyl side chain. Steady state kinetic experiments were once again used to determine dCTP and dATP incorporation efficiencies opposite OdG and its analogues with Ile716Met BF and Ile716Ala BF. Again, dG, dI, CldG, and BrdG incorporation efficiencies with dATP were not measured, as these analogues lack an N7-hydrogen necessary for the formation of a base pair to dA. The parameters of dCTP and dATP incorporations with Ile716Met BF and Ile716Ala can be seen in Table 3. Comparing the results between the two mutant enzymes, and WT BF, offers key findings and greater insight into the influence of the Ile716 residue.
Table 3. Reaction parameters for steady state single nucleotide incorporation of dCTP or dATP with Ile716Met. a

<table>
<thead>
<tr>
<th>X:dNTP</th>
<th>Vmax (%) min⁻¹</th>
<th>Km dNTP (µM)</th>
<th>Vmax/Km dCTP (µM⁻¹)</th>
<th>Vmax/Km dATP b</th>
<th>Km dNTP (µM)</th>
<th>Vmax/Km dCTP (µM⁻¹)</th>
<th>Vmax/Km dATP b</th>
</tr>
</thead>
<tbody>
<tr>
<td>dG:dCTP</td>
<td>20 ± 5</td>
<td>0.02 ± 0.00</td>
<td>1200 ± 440</td>
<td>16 ± 1</td>
<td>2.8 ± 0.5</td>
<td>5.6 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>CldG:dCTP</td>
<td>310 ± 70</td>
<td>330 ± 70</td>
<td>0.95 ± 0.29</td>
<td>57 ± 17</td>
<td>350 ± 70</td>
<td>0.16 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>BrdG:dCTP</td>
<td>220 ± 50</td>
<td>410 ± 100</td>
<td>0.53 ± 0.18</td>
<td>86 ± 13</td>
<td>300 ± 100</td>
<td>0.29 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>CdG:dCTP</td>
<td>69 ± 1</td>
<td>1.2 ± 0.2</td>
<td>65 ± 16</td>
<td>5.9</td>
<td>32 ± 9</td>
<td>26 ± 9</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>CdG:dATP</td>
<td>130 ± 20</td>
<td>12 ± 3</td>
<td>11 ± 3</td>
<td>33 ± 1</td>
<td>75 ± 10</td>
<td>0.44 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>OdG:dCTP</td>
<td>260 ± 10</td>
<td>210 ± 40</td>
<td>1.3 ± 0.2</td>
<td>1.2</td>
<td>4.8 ± 0.2</td>
<td>210 ± 60</td>
<td>0.023 ± 0.006</td>
</tr>
<tr>
<td>OdG:dATP</td>
<td>83 ± 6</td>
<td>79 ± 17</td>
<td>1.1 ± 0.0</td>
<td>1.2</td>
<td>6.0 ± 0.6</td>
<td>260 ± 40</td>
<td>0.0049 ± 0.0027</td>
</tr>
<tr>
<td>SdG:dCTP</td>
<td>16 ± 2</td>
<td>280 ± 60</td>
<td>0.057 ± 0.013</td>
<td>0.24</td>
<td>24 ± 3</td>
<td>560 ± 110</td>
<td>0.043 ± 0.010</td>
</tr>
<tr>
<td>SdG:dATP</td>
<td>17 ± 3</td>
<td>73 ± 11</td>
<td>0.24 ± 0.05</td>
<td>0.10 ± 0.00</td>
<td>66 ± 16</td>
<td>0.0018 ± 0.0005</td>
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<tr>
<td>OdI:dCTP</td>
<td>370 ± 50</td>
<td>190 ± 40</td>
<td>1.9 ± 0.5</td>
<td>0.11</td>
<td>13 ± 2</td>
<td>410 ± 70</td>
<td>0.031 ± 0.010</td>
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<tr>
<td>OdI:dATP</td>
<td>70 ± 7</td>
<td>4.0 ± 0.8</td>
<td>17 ± 4</td>
<td>8.6 ± 0.8</td>
<td>190 ± 30</td>
<td>0.045 ± 0.009</td>
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<tr>
<td>SdI:dCTP</td>
<td>14 ± 2</td>
<td>310 ± 50</td>
<td>0.046 ± 0.006</td>
<td>0.027</td>
<td>1.6 ± 0.2</td>
<td>210 ± 17</td>
<td>0.0075 ± 0.0012</td>
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<tr>
<td>SdI:dATP</td>
<td>47 ± 7</td>
<td>27 ± 5</td>
<td>1.7 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>220 ± 30</td>
<td>0.0053 ± 0.0010</td>
<td></td>
</tr>
<tr>
<td>dI:dCTP</td>
<td>9 ± 1</td>
<td>0.060 ± 0.010</td>
<td>1500 ± 300</td>
<td>0.30 ± 0.00</td>
<td>95 ± 26</td>
<td>6.6 ± 1.8</td>
<td></td>
</tr>
</tbody>
</table>

a: Vmax and Km values are reported as the average ± sd.
b: Incorporation preference = (Vmax/Km)dCTP / (Vmax/Km)dATP
When looking at the overall activity of the enzymes, Ile716Met BF was found to be generally about 10-fold more active, while Ile716Ala was about 100-fold less active than WT BF (comparing dCTP incorporation opposite OdG). The increased activity of Ile716Met BF in comparison to WT BF suggests that replacing Ile716 with a straight-chained Met residue changes the active site, which may confer changes in the polymerase’s ability to conform to the cognate base pair shape. When Ile716 is changed to an Ala residue, the shape of the active site likely becomes less tight. This change is associated with decreased relative activity of the polymerase, as Ile716Ala BF is likely less able to conform to the cognate base pair shape.

Turning to individual incorporation experiments, Ile716Met appears less tolerant to steric bulk at the C8-position during dCTP incorporation in comparison to WT BF. This is evident when comparing dCTP incorporation opposite dG to CldG and BrdG with the two polymerases (Tables 2, 3 and Figures 7, 9). WT BF exhibits 400- and 1000-fold decreases in dCTP incorporation efficiency opposite CldG and BrdG, respectively, as compared to opposite dG. With Ile716Met BF, incorporations opposite CldG and BrdG

Figure 9. Graphical depiction of steady state data collected for the incorporation of dCTP and dATP opposite a template XdG with Ile716Met BF. Conditions at 37 °C, 0.4 μM DNA and 0.1 nM Ile716Met BF.

Figure 10. Graphical depiction of steady state data collected for the incorporation of dCTP and dATP opposite a template XdG with Ile716Ala BF. Conditions at 37 °C, 0.4 μM DNA and 1 nM Ile716Ala BF.
were 1300- and 2300-fold less efficient than opposite dG, a greater decrease than was seen with WT BF. These results are somewhat surprising, as one may expect that the straight chain methionine residue would clash less with a bulky C8-atom than the branched chain isoleucine. It is possible that the mutation to a methionine residue induces a different conformation or interactions in the active site that influences the enzyme’s activity, however further structural studies would be necessary to confirm this point.

Unlike Ile716Met, Ile716Ala appears to be more tolerant than WT BF to large atoms off C8-position (Tables 2, 3 and Figures 7, 10). For example, with Ile716Ala BF, dCTP incorporations opposite CldG and BrdG decrease by only 35- and 19-fold, respectively, as compared to opposite dG. Thus, during dCTP incorporation, Ile716Ala seems more tolerant of large atoms like chlorine and bromine in the major groove, likely due to the change in shape of the active site. Overall, these results suggest that the residue at this position plays a key role in the enzymes ability to fit tightly to the substrate, thus influencing overall incorporation efficiencies and activity.

Another similarity that can be seen across the polymerases is the effect of the C2-amine during dATP incorporation. Experiments with all three enzymes provide evidence for a clash between the C2-amine and Ile716. This is apparent when comparing dATP incorporation efficiencies opposite OdG and OdI, and opposite SdG and SdI. Similar to WT BF, with Ile716 Met BF and Ile716Ala BF, there were 17- and 9-fold increases, respectively in efficiency opposite OdI as compared to OdG, and 7- and 3-fold increases, respectively, opposite SdI as compared to SdG. Given these results, the C2-amine group appears to play a key role in determining dATP incorporation efficiency.
Looking more generally at dATP incorporation opposite OdG offers insight on how the major groove mutation at Ile716 can be a factor when comparing activity opposite nucleotides with changes at the C8-position in the minor groove. For example, as compared to opposite CdG, WT BF exhibits 3- and 16-fold decreases in dATP incorporation opposite OdG and SdG, respectively, while Ile716Met BF and Ile716Ala BF showed 11- and 90-fold decreases, respectively, opposite OdG, and 46- and 244-fold decreases, respectively, opposite SdG. While there is a decrease in dATP incorporation efficiency with increasing steric bulk at this C8-position, this likely cannot be accounted for by clashing in the minor groove, as Tyr714 is conserved across all three enzymes. Instead, it appears that looser, more open active sites may be more sensitive to large atoms in the minor groove. In summary, steric bulk at the C8-position may affect dATP incorporation efficiency, and thus dCTP/dATP incorporation preference.

When comparing dNTP incorporation preference opposite OdG, there were differences between all three polymerases. This is due, in part, to the active site interactions between the substituents at the C8- and C2-positions and the amino acid at the 716 position. As mentioned, WT BF exhibits 3-fold preferential incorporation of dATP opposite OdG. When the Ile residue is changed to the straight side chain methionine residue in Ile716Met BF, a more equal preference (approximately 1.2-fold preference for dCTP) is observed. Finally, in the case of Ile716Ala BF, in which isoleucine is replaced by an even smaller residue, OdG instead preferentially directs dCTP incorporation, by 4.7-fold. The differences in dCTP/dATP incorporation preferences in Ile716Met BF and Ile716Ala BF and WT BF appear to be a combination of tolerance during dCTP incorporation and added sensitivity to dATP incorporation with
large C8-atoms when the active site is more open or loose. In summary, Ile716 plays a key role in the dCTP/dATP incorporation ratio and consequently the mutagenic potential of this lesion.
Conclusions

The importance of Ile716 to the mutagenic potential of OdG was studied by comparing WT BF, Ile716Ala, and Ile716Met BF. More specifically, the incorporation efficiencies of dCTP and dATP opposite dG, OdG, and seven of their analogues were tested with both enzymes in order to examine the importance of clashing between Ile716 and either the C2-amine or C8-oxygen of OdG. Opposite OdG, WT BF has a preference for dATP incorporation, likely due, in part, to the large size of the Ile716 side chain clashing with the C8-oxygen of OdG when it is in the anti-conformation and paired with dCTP. Consistent with these results, when the branched Ile716 is mutated to the straight chain Met, there is a more equal preference between dCTP and dATP incorporation opposite OdG, and when Ile716 is mutated to Ala, a preference for dCTP incorporation results. Furthermore, dCTP incorporation reactions opposite ClG and BrdG and OdG and SdG were less efficient than opposite dG and CdG, respectively, with WT BF and Ile716Met, but were more equal with the Ile716Ala mutant. During dATP incorporation, all three enzymes provided evidence for a clash between the amino acid at Ile716 and the C2-amine of OdG. In summary, the Ile716Met mutant was similar overall to WT BF in terms of its tolerance to steric and electronic variations from the normal Watson Crick base pairs, while the Ile716Ala mutant was more tolerant to such changes. In summary, this study provides evidence for the role of Ile716 in the mutagenic potential of OdG.
Acknowledgments

I wish to thank Dr. Michelle Hamm for her direction and patience over my three years working in her lab, as well as my fellow lab mates and funding sources from the NSF-RUI program, the Research Corporation Inc., and the University of Richmond.
Works Cited


