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The Importance of Ile716 toward the Mutagenicity of 8-Oxo-2'-deoxyguanosine with Bacillus Fragment DNA Polymerase

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Abstract

8-oxo-2'-deoxyguanosine (OdG) is a prominent DNA lesion that can direct the incorporation of dCTP or dATP during replication. As the latter reaction can lead to mutation, the ratio of dCTP/dATP incorporation can significantly affect the mutagenic potential of OdG. Previous work with the A-family polymerase BF and seven analogues of OdG identified a major groove amino acid, Ile716, which likely influences the dCTP/dATP incorporation ratio opposite OdG. To further probe the importance of this amino acid, dCTP and dATP incorporations opposite the same seven analogues were tested with two BF mutants, I716M and I716A. Results from these studies support the presence of clashing interactions between Ile716 and the C8-oxygen and C2-amine during dCTP and dATP incorporations, respectively. Crystallographic analysis suggests that residue 716 alters the conformation of the template base prior to insertion into the active site, thereby affecting enzymatic efficiency. These results are also consistent with previous work with A-family polymerases, which indicate they have tight, rigid active sites that are sensitive to template perturbations.

1. Introduction

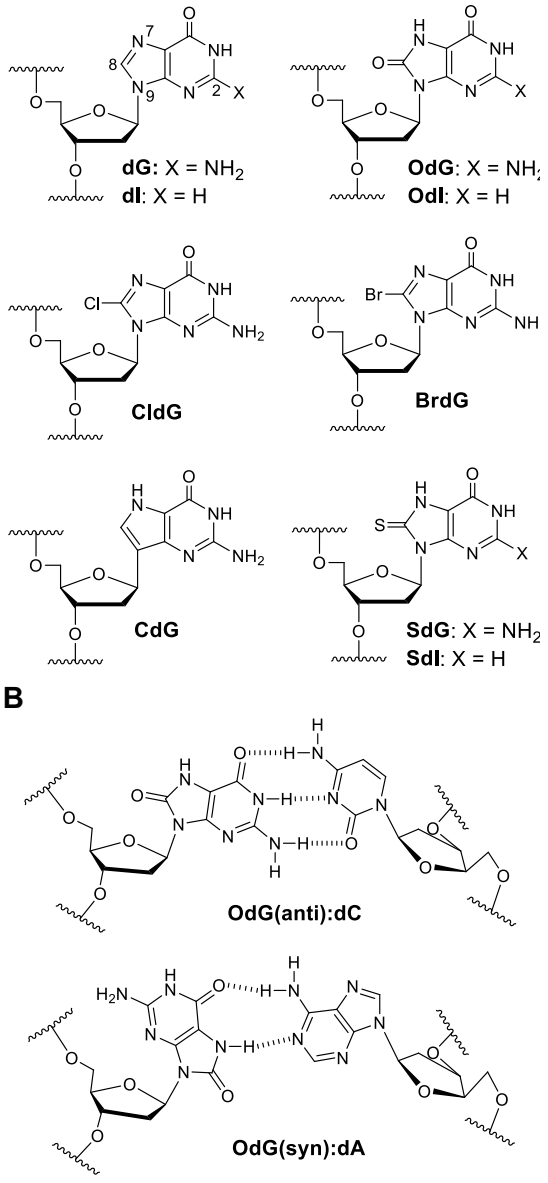
8-oxo-2'-deoxyguanosine (OdG) is a potent promutagen that arises when reactive oxygen species encounter 2'-deoxyguanosine (dG; Scheme 1A).[1] OdG lesions are commonly formed in mammalian cells and have been linked to ageing, cancer, and autoimmune diseases.[2-4] Due to steric and electronic changes in the imidazole ring as compared to dG, OdG can form stable base pairs to both 2'-deoxycytidine (dC) and 2'-deoxyadenosine (dA).[5] OdG uses the anti-conformation when base pairing with dC to form a base pair that is similar to a dG:dC base pair.[6] Alternatively, OdG uses the syn-conformation when base paring with dA to form a structure that is similar to a dT:dA base pair (Scheme 1B).[7]

Since OdG:dC and OdG:dA base pairs both mimic natural base pairs and have similar stabilities,[8] polymerases often have trouble distinguishing between them. Consequently, a polymerase may pair a template OdG lesion with either dCTP or dATP. The incorporation of a

dATP opposite OdG can lead to a dG→dT transversion mutation. Thus, the promutagenic character of OdG is highly dependent on the tendency of a given polymerase to pair OdG with dCTP or dATP.

Previous research has shown that the dCTP/dATP incorporation ratio varies widely within and between polymerase families,[9-11] suggesting that differences in individual polymerase insertion sites can have a strong influence over the mutagenic potential of OdG. To further probe this idea, our lab previously studied two A-family polymerases that are largely homologous, but have differing dCTP/dATP incorporation ratios opposite OdG. Klenow Fragment from *Escherichia coli* DNA polymerase I (KF) that lacks the 3'-exonuclease site (KF-exo) prefers to insert dCTP opposite OdG,[12] while the large fragment from *Bacillus stearothermophilus* polymerase I (BF), which also lacks a proofreading exonuclease site, prefers dATP incorporation.[13]

In order to tease out subtle active site interactions that may lead to the different incorporation preferences of KF-exo and BF, we tested dCTP and dATP incorporations opposite nucleotide analogues that differed from dG or OdG at the C8- and/or C2-positions.[14] These analogues included (i) 8-chloro-2'-deoxyguanosine (CldG) and 8-bromo-2'-deoxyguanosine (BrdG), which contain relatively large atoms off C8 similar to that of OdG, but otherwise mimic dG, (ii) 9-deaza-2'-deoxyguanosine (CdG) and 8-thio-2'-deoxyguanosine (SdG), which contain the N7-hydrogen used when pairing to dA, but differ from OdG in their steric and electronic properties at C8, and (iii) 2'-deoxyinosine (dI), 8-oxo-2'-deoxyinosine (OdI), and 8-thio-2'-deoxyinosine (SdI), which all lack an C2-exocyclic amine, but otherwise mimic dG, OdG, and SdG, respectively (Scheme 1A).



Scheme 1. (A) dG, OdG and the seven analogues used in this study. (B) When paired to dC, OdG is in the anti-conformation and when paired to dA, OdG is in the syn-conformation.

Our work with these seven dG/OdG analogues indicated that both KF-exo and BF are sensitive to groups that extend outside the cognate Watson−Crick (W−C) base pair shape. This finding is consistent with previous work that showed A-family polymerases have tight, rigid active sites that are sensitive to steric perturbations.[15, 16] The inverted dCTP/dATP incorporation ratios opposite OdG between the two polymerases appeared to derive from the different extents of this sensitivity. BF is much less tolerant of changes at C8 during dCTP incorporation, leading to a preference for ATP incorporation opposite OdG. Using available crystal structures, we modeled OdG:dCTP and OdG:dATP in the insertion site of BF, and found a potential clash in the major groove between Ile716 and the C8-oxygen and C2-amine during dCTP and dATP incorporation, respectively (Figure 1). In KF-exo, the corresponding amino acid is Met768. The straight and more flexible chain of Met[17, 18] as opposed to the branched chain of Ile may help explain why BF is more sensitive to perturbations at C8 than KF-exo during dCTP incorporation.

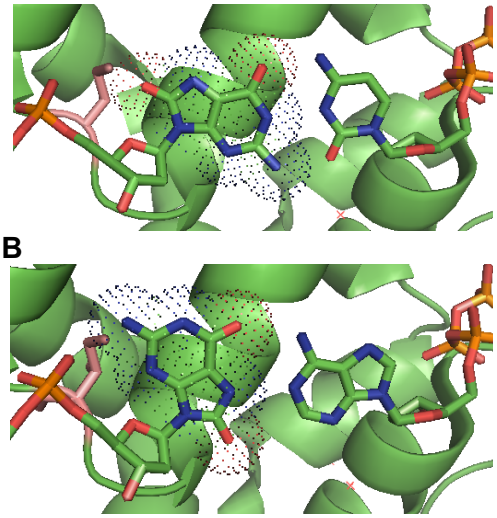


Figure 1. PDB 1LV5 modelled with (A) OdG:dCTP (from PDB 1U47) or (B) OdG:dATP (from PDB 1U49) in the insertion site using Coot Version 0.6.1, and displayed using PyMOL. Ile716 is in salmon and the dotted spheres represent the Van der Waal radii of the 8-oxoguanine base. The rest of the DNA duplex is hidden for ease of viewing.

To further test for clashing with Ile716 and its effect on incorporations opposite OdG, we expressed and purified WT BF and two BF mutants, I716M and I716A. The methionine mutant alters BF to be more like KF-exo, which prefers dCTP incorporation opposite OdG, while the alanine mutant removes the potentially clashing side chain altogether. By testing dCTP and dATP incorporations opposite dG, OdG, and the seven analogues with each mutant BF, we hoped to gain additional insight into the importance of Ile716 to OdG mutagenicity.

2. Materials and Methods

2.1 Polymerase Mutation, Expression, and Purification

The gene sequence encoding residues 297-876 of DNA polymerase I from *Bacillus stearothermophilus*[19] was synthesized and cloned into pET21a immediately behind the NdeI site and its start codon (Genscript, Piscataway, New Jersey). The mutations of isoleucine 716 to methionine (I716M) or alanine (I716A) were generated by site-directed mutagenesis (Genscript). The expression plasmids were transformed into BL21(DE3)pLysS *Escherichia coli* cells and grown to log phase. Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside when the culture absorbance at 600 nanometers reached ~0.6. Culture was shaken for 4 hours at 37°C, then harvested by pelleting in a centrifuge. The bacteria were frozen at -20°C overnight, thawed, then lysed with B-PER detergent (Thermo Fisher Scientific, Waltham, Massachusetts), lysozyme (Hampton Research, Aliso Viejo, California) and Pierce Universal Nuclease for Cell Lysis (Thermo

Fisher Scientific) according to manufacturer's instructions. Cell lysates were heated to 65°C for 10 minutes to denature some of the cellular proteins, then separated by centrifugation at 15,000 *x* g for 5 minutes at 4°C (This step was omitted for I716A BF due to thermal instability). Purification of BF proteins via ion exchange chromatography and heparin sulfate affinity chromatography was performed as previously described.[5]

2.2 Oligonucleotide Synthesis and Purification

Template oligonucleotides were 5'-d(TCACXCTGCTGTCGG)-3', where X is dG, BrdG, CldG, CdG, OdG, SdG, dI, OdI or SdI, and primer oligonucleotides were 5'-d(CCGACAGCAG)-3'. Unmodified oligonucleotides were purchased from IDT DNA, while those containing OdG and BrdG were purchased from Midland Inc. Oligonucleotides containing CdG,[20] SdG,[21] CldG,[22] OdI,[14, 23] and SdI[14] were synthesized and characterized as previously described. All oligonucleotides were purified by 20% denaturing PAGE and HPLC as previously described.[14]

2.3 Radiolabeling of Primers

Primers were radiolabeled using Optikinase (USB) and γ-^{32}P-ATP (MP Biomedical) and purified using mini Quick Spin Oligo Columns (Roche) for a final concentration of 4 μM.

2.4 Steady State Insertion Experiments

Solutions containing Tris-HCl, $MgCl_2$, DTT, BSA, template, and primer were heated at 90°C for three minutes before slow cooling to room temperature. BF was then added so that the final concentrations in the solution were 100 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 2 mM DTT, 5 μg/ml BSA, 0.2 μM template, 0.2 μM ^{32}P-radiolabeled primer, and 0.4 nM WT BF, 0.2 nM BF I716M or 20 nM BF I716A in 10% glycerol. 5 μl of the above solution was incubated at 37°C for 5 minutes and then added to 5 μL of a 2x dNTP solution (that had also been incubated at 37°C for 5 minutes) containing 20 mM NaCl and the appropriate dNTP concentration. After the appropriate time, reactions were stopped with 20 μL of a solution containing 95% formamide, 20 mM EDTA, and 0.0025% each of bromophenol blue and xylene cyanol.

Product oligonucleotides were separated from starting oligonucleotides using 20% denaturing PAGE. The resulting gel was dried, exposed to a storage phosphor screen (Amersham) overnight, and visualized using a Storm 860 Phosphorimager (Amersham). Steady state kinetic experiments were run so that ≤ 20% of the reaction had progressed, and included seven different dNTP concentrations. Reagent and product bands were quantified using ImageQuant 8.0. Individual Michaelis-Menten curves were generated with Kaleidagraph 4.5 or SigmaPlot 9.0 using values obtained from the averaging of three experiments. k_{cat} and K_m ± standard deviation were obtained from the average of at least three Michaelis-Menten experiments. Overall activity was provided by the specificity constant (k_{cat}/K_m) and overall error was determined by propagation of the individual errors for k_{cat} and K_m.

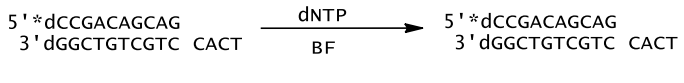
2.5 Crystallization and Structure Determination

Concentrated BF I716M was mixed with annealed double-stranded DNA containing OdG in a 5'-overhang (template strand 5'-d(ATC[OdG]GCGTGATCG)-3' and primer strand 5'-d(CGATCACGC)-3') at a final concentration of 0.11 mM BF and 0.22 mM DNA. The complex was crystallized by hanging drop vapor diffusion with a well solution of 44% ammonium sulfate, 0.1 M 2-(N-morpholino)ethanesulfonic acid pH 6.0, 10 mM magnesium sulfate, and 1% 2-methyl-2,4-pentanediol. A crystal was soaked in the well solution plus 21% sucrose to cryoprotect prior to freezing in liquid nitrogen. X-ray diffraction data were collected using a Rigaku MicroMax-007HF copper rotating anode generator and a Dectris Eiger R 4M detector. Phases were determined by molecular replacement using Phaser[24] and Protein Data Bank file 1L3S.pdb[25] as a model. The structure was adjusted and refined using Coot[26] and Refmac5.[27]

3. Results and Discussion

The efficiencies of dCTP and dATP incorporations opposite dG, OdG, and the seven OdG analogues were quantified for WT, I716M, and I716A BF polymerases using single-nucleotide insertion experiments and steady state kinetics (Tables 1 and 2).[28] Oligonucleotide templates (15 nucleotides long) were paired with 5'-radiolabeled primers (10 nucleotides long) so that the incoming dNTP was added to the primer opposite a template dG, OdG, or one of the analogues. Based on previous work with BF,[14] qualitative results with the two mutant BF polymerases (Supplementary data Figure S1), and the known stability of OdG:dC and OdG:dA base pairs, only dCTP and dATP incorporations were quantified. Furthermore, dATP incorporations were not quantified opposite dG, CldG, BrdG and dI. These four reactions were all quite inefficient (Supplementary data Figure S1) and all four nucleotides lack a N7-hydrogen; thus, the resulting base pairs would not mimic OdG:dATP, limiting their relevance.

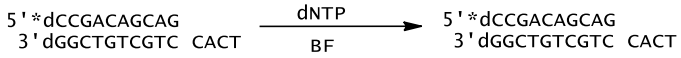
Table 1. Kinetic parameters for steady state incorporations of dCTP or dATP with WT BF.



X:dNTP	k_{cat} (min^{-1})[a]	K_m dNTP (μM)[a]	k_{cat}/K_m (min^{-1} μM^{-1})	dCTP/dATP[b]
dG:dCTP	120 ± 30	0.43 ± 0.09	270 ± 80	
CldG:dCTP	330 ± 50	490 ± 30	0.67 ± 0.11	
BrdG:dCTP	160 ± 50	620 ± 160	0.26 ± 0.10	
CdG:dCTP	430 ± 90	69 ± 10	6.2 ± 1.6	2.8
CdG:dATP	360 ± 100	170 ± 20	2.2 ± 0.7	
OdG:dCTP	32 ± 7	370 ± 50	0.085 ± 0.019	0.17
OdG:dATP	38 ± 11	77 ± 26	0.49 ± 0.22	
SdG:dCTP	5.5 ± 1.0	370 ± 50	0.015 ± 0.003	0.32
SdG:dATP	13 ± 4	280 ± 80	0.046 ± 0.018	
dI:dCTP	14 ± 5	0.057 ± 0.013	240 ± 100	
OdI:dCTP	260 ± 50	600 ± 120	0.43 ± 0.12	0.20
OdI:dATP	490 ± 70	220 ± 30	2.2 ± 0.4	
SdI:dCTP	18 ± 4	140 ± 30	0.13 ± 0.04	0.27
SdI:dATP	45 ± 7	94 ± 17	0.48 ± 0.11	

a. Average ± standard deviations calculated from three or more experiments. b. dCTP/dATP = $(k_{cat}/K_m)_{dCTP}/(k_{cat}/K_m)_{dATP}$.

Table 2. Kinetic parameters for steady state incorporations of dCTP or dATP with I716M and I716A BF.



	I716M BF				I716A BF			
X:dNTP	k_{cat} (min^{-1})[a]	K_m dNTP (μM)[a]	k_{cat}/K_m (min^{-1} μM^{-1})	dCTP/dATP[b]	k_{cat} (min^{-1})[a]	K_m dNTP (μM)[a]	k_{cat}/K_m (min^{-1} μM^{-1})	dCTP/dATP[b]
dG:dCTP	200 ± 50	0.016 ± 0.004	12000 ± 4000		5.4 ± 1.2	0.69 ± 0.15	7.8 ± 2.4	
CldG:dCTP	3100 ± 700	330 ± 70	9.5 ± 2.8		57 ± 17	350 ± 70	0.16 ± 0.06	
BrdG:dCTP	2100 ± 500	410 ± 100	5.3 ± 1.8		85 ± 13	300 ± 120	0.28 ± 0.12	
CdG:dCTP	700 ± 100	1.1 ± 0.2	650 ± 160	5.9	32 ± 9	26 ± 9	1.2 ± 0.5	2.7
CdG:dATP	1300 ± 100	12 ± 3	110 ± 30		33 ± 1	75 ± 10	0.44 ± 0.06	
OdG:dCTP	2600 ± 40	210 ± 40	13 ± 2	1.3	15 ± 3	180 ± 30	0.083 ± 0.022	3.2
OdG:dATP	830 ± 60	80 ± 17	10 ± 2		2.9 ± 0.4	110 ± 10	0.026 ± 0.004	
SdG:dCTP	150 ± 20	260 ± 60	0.57 ± 0.14	0.24	1.5 ± 0.4	180 ± 10	0.0083 ± 0.0023	1.9
SdG:dATP	170 ± 30	73 ± 11	2.4 ± 0.5		0.51 ± 0.05	120 ± 10	0.0043 ± 0.0006	
dI:dCTP	91 ± 6	0.0063 ± 0.0014	15000 ± 3000		3.9 ± 0.8	0.94 ± 0.30	4.2 ± 1.6	
OdI:dCTP	3700 ± 500	200 ± 40	19 ± 5	0.11	8.0 ± 1.8	460 ± 120	0.017 ± 0.006	0.39
OdI:dATP	700 ± 70	4.0 ± 0.8	170 ± 40		8.3 ± 2.1	190 ± 20	0.044 ± 0.004	
SdI:dCTP	140 ± 20	310 ± 30	0.46 ± 0.10	0.27	1.4 ± 0.2	280 ± 60	0.0050 ± 0.0013	0.94
SdI:dATP	470 ± 70	30 ± 5	17 ± 4		0.90 ± 0.09	170 ± 30	0.0053 ± 0.0011	

a. Average ± standard deviations calculated from three or more experiments. b. dCTP/dATP = $(k_{cat}/K_m)_{dCTP}/(k_{cat}/K_m)_{dATP}$.

3.1 Structure and activity of the BF mutants

Interestingly, as compared to WT BF, reactions with I716M BF were ~40-fold more efficient overall, while the reactions with I716A BF were ~30-fold less efficient overall (Tables 1 and 2). The former result is in line with our previous work with KF-exo and BF;[14] the Met containing KF-exo was also overall more efficient than the Ile containing BF. Additionally, the lower efficiency of I716A BF is correlated with its tendency to denature during a 65°C incubation during purification, suggesting that the alanine mutation destabilizes the polymerase structure.

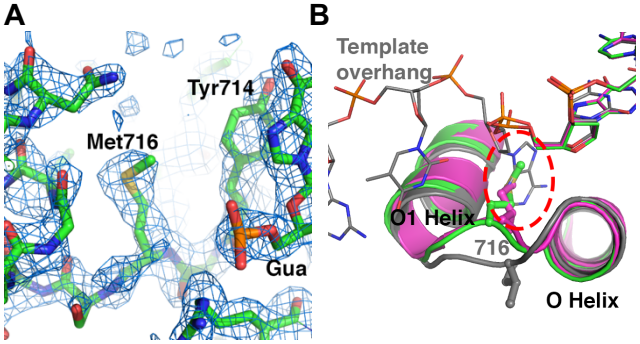


Figure 2. Crystal structure of I716M BF in complex with DNA. (A) The $2F_O$-F_C electron density map at 1.2σ is displayed over the model in green sticks. Strong electron density for the DNA template strand ends at guanine 5 (Gua). (B) The O-O1 loop of I716M BF (green) adopts a similar conformation to a BF-blunt-ended DNA complex (1L3V; magenta), not a complex containing a template adenosine (1L3S; grey). The preinsertion site is outlined by a red dotted oval.

To further examine the high efficiency of I716M BF relative to WT BF, we solved the X-ray crystal structure of I716M BF-DNA[OdG] complex to 2.2Å resolution (Supplementary data Table S1). The overall structure of the enzyme-

DNA complex is nearly identical to the WT BF-DNA complex (1L3S.pdb)[25] and the electron density for Met716 is clearly seen in the $2F_O$-F_C map (Figure 2A). Interestingly, instead of fitting neatly into a hydrophobic pocket like Ile716 in WT BF (grey in Figure 2B), the Met side chain in I716M BF (green in Figure 2B) sticks directly into a gap between the O and O1 helices. This gap (red oval in Figure 2B) has been labeled the "preinsertion site"[25] based on structural data with WT BF that indicated the template base (grey adenine in Figure 2B) can occupy this site as it awaits interaction with the incoming nucleotide. The occlusion of the preinsertion site by Met716 in I716M BF coincides with the absence of electron density beyond the OdG phosphorus atom and through the rest of the template overhang, indicating that these nucleotides are unconstrained and can adopt multiple conformations. In addition, the backbone of the loop between the O and O1 helices in the I716M BF structure is indistinguishable from a WT BF-DNA complex without a template overhang (1L3V.pdb; magenta in Figure 2B),[25] suggesting that the conformations of the template overhang and the O-O1 loop are correlated. Based on these structural data, the increased activity of I716M BF as compared to WT BF may be in part due to a difference in preinsertion site occupancy prior to catalysis. In WT BF, it appears the template base outcompetes Ile716 and enters the preinsertion site, while in I716M BF, it is possible the template base skips over the Met716-filled preinsertion site and readily enters the insertion site (active site), thereby improving catalysis.

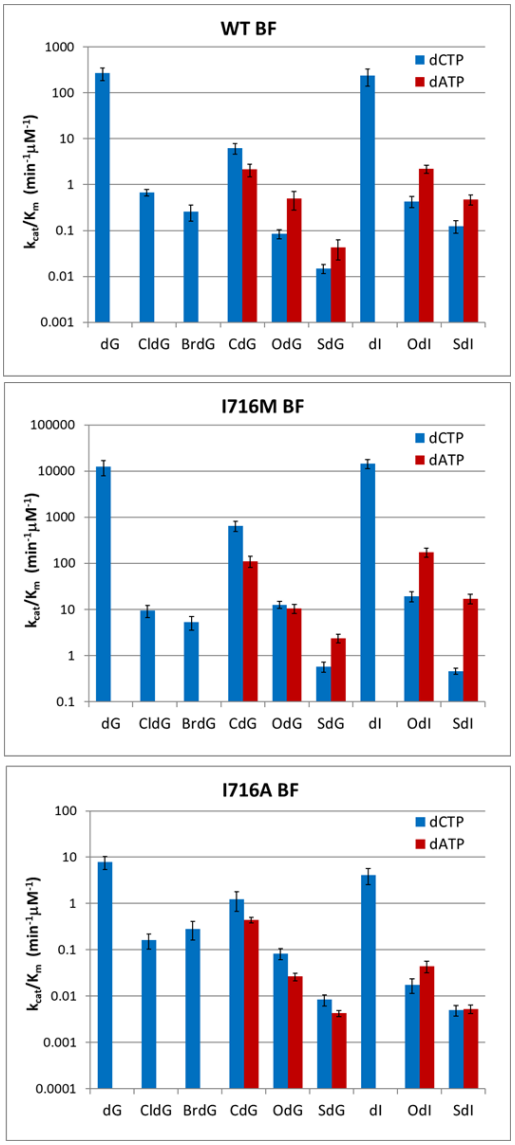


Figure 3. Graphical representations, with a logarithmic y-axis, of the specificity constants ($k_{cat.}/K_m$) determined with WT, I617M, and I716A BF as shown in Tables 1 and 2.

3.2 dCTP incorporations

When looking at the efficiencies of dCTP incorporations opposite dG, OdG, and the analogues with the three BF polymerases, many similar trends are observed (Figure 3). Analogous to our previous work with BF,[14] dCTP incorporation efficiencies were reduced opposite all analogues that contained a large atom off C8, though the extent of the reduction varied. For example, dCTP incorporation efficiencies opposite CldG and BrdG as compared to dG were reduced more with WT and I716M BF (between 400- and 2300-fold) than with I716A BF (between 30- and 50-

fold). These results suggest that during dCTP incorporation, WT and I716M BF are more sensitive than I716A BF to imidazole ring perturbations and provide additional evidence for a destabilizing interaction between Ile716 and a large atom off C8 (Figure 1A).

The efficiencies of dCTP incorporations were also reduced opposite CdG, OdG and SdG as compared to dG; these reaction efficiencies were most reduced for WT BF (43-, 3100-, and 18,000-fold, respectively), overall less reduced for I716M BF (19-, 1000-, and 21,000-fold, respectively) and least reduced for I716A BF (6-, 90-, and 900-fold, respectively). Thus, once again, the I716A BF mutant was the most tolerant to large atoms off the C8-position of a template base and provides further evidence that A-family polymerases like BF have tight, rigid active sites that are sensitive to changes from the cognate base pair shape.[15] The data are also consistent with WT BF being the most sensitive to the electronic changes in the imidazole ring that come with the presence of an N7-hydrogen as found in OdG. Despite containing similarly sized or smaller atoms off C8, dCTP was often incorporated less efficiently opposite OdG (and SdG) as compared to CldG and BrdG, though the difference was most pronounced with WT BF.

3.3 dATP incorporations

During dATP incorporation, when OdG is in the syn-conformation, the C8-position is in the minor groove and lies in close contact with a conserved Tyr714.[13, 29] Similar to our previous results with BF, dATP incorporations were most efficient opposite CdG and increasingly less efficient opposite OdG and SdG as the C8-atom grows larger. Interestingly, the efficiency reductions were similar for all three enzymes, likely due to the similar minor grooves of the three enzymes.

Turning back to the major groove, during dATP incorporation, the C2-amine (instead of a C8-atom) lies in close proximity to the polymerase and can clash with Ile716 (Figure 1B). Previous results with our analogues are consistent with such a clash; removal of the C2-amine from OdG and SdG (to form OdI and SdI, respectively) lead to increased dATP incorporation efficiencies.[14] In the studies herein, efficiency increases between OdI/SdI as compared to OdG/SdG were observed with both WT and I716M BF (increases of 5-16 fold), but not with I716A BF (increases of less than 2-fold). This difference is most easily explained by the mutation of Ile716 to Ala already eliminating any clash between Ile716 and the C2-amine so that removal of the C2-amine does not lead to additional activity. However, it is of note that the similar activity opposite OdG/SdG and OdI/SdI with I716A is due to particularly poor dATP incorporation opposite OdI and SdI instead of increased efficiency opposite OdG and SdG, which was previously observed during similar experiments with the Y-family polymerase, Dpo4 (where the C2-amine clashes with Arg332).[30]

3.4 dCTP/dATP incorporation ratios

Finally, in this study, WT BF had a roughly 6-fold preference for dATP incorporation opposite OdG, similar to previous studies that have shown a 9- and 4.5- fold preference.[13, 14] For both the BF mutants, there was an increased preference for dCTP incorporation opposite OdG. I716M showed a similar dCTP/dATP incorporation ratio, and I716A showed a roughly 3-fold preference for dCTP incorporation. Based on the results previously discussed, it appears these differences are primarily due to lesser tolerance of WT and I716M BF for electronic changes in the imidazole ring and large

atoms off C8 during dCTP incorporation. Thus, it seems that during these reactions, the mutation of Ile716 to alanine may open or loosen the normally rigid active site of this A-family polymerase, increasing the tolerance for variations in the major grove of the template nucleotide.

4. Conclusions

In summary, this work builds on previous work with OdG and BF and provides additional evidence that Ile716 plays an important role during OdG replication, increasing the mutagenic potential of OdG, but also limiting the efficiency of both dCTP and dATP incorporations opposite this common DNA lesion. It also supports the wider theory that A-family polymerases have constrained active sites that conform to the shape of Watson-Crick base pairs, making them sensitive to steric variations. Furthermore, Ile716 plays an important role during undamaged DNA replication by creating the preinsertion site pocket into which the template base slots prior to encountering the incoming nucleotide. Thus, DNA synthesis efficiency can be tuned positively or negatively by mutating Ile716. Finally, this research also supports previous work that indicates individual amino acids can play an important role in effecting OdG mutagenesis, replication fidelity, and polymerase efficiency.[31-37]

Conflict of Interest

The authors have no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version.

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