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DNA Polymerase Beta Cancer-Associated Variant I260M Exhibits Nonspecific Selectivity Towards β - γ Bridging Group of the Incoming dNTP

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Abstract

The hydrophobic hinge region of DNA polymerase β (pol β) is located between the fingers and palm subdomains. The hydrophobicity of the hinge region is important for maintaining the geometry of the binding pocket and for selectivity of the enzyme. Various cancer-associated pol β variants in the hinge region have reduced fidelity resulting from decreased discrimination at the level of dNTP binding. Specifically, I260M, a prostate cancer-associated variant of pol β , has been shown to have reduced discrimination during dNTP binding and also during nucleotidyl transfer. To test whether fidelity of the I260M variant is dependent on leaving group chemistry, we employed a tool-kit comprising dNTP bisphosphonate analogues modified at the β - γ bridging methylene to modulate leaving group (pCXYp mimicking PPI) basicity. Construction of LFER plots for the dependence of $\log(k_{\text{pol}})$ on the leaving group $\text{p}K_{\text{a}4}$ revealed that I260M catalyzes dNMP incorporation with a marked negative dependence of leaving group basicity, consistent with a chemical transition state, during both correct and incorrect incorporation. Additionally, we provide evidence that the I260M fidelity is altered in the presence of some of the analogues, possibly resulting from a lack of coordination between the fingers and palm subdomains in the presence of the I260M mutation.

Graphical abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Keywords

Cancer-related mutation; base excision repair; DNA polymerase β ; hydrophobic hinge region; β , γ -methylene biphosphonate nucleotide substrate analogues; linear free energy relationship; fidelity

DNA Polymerase β (pol β) is an essential component of the base excision repair (BER) pathway, important for gap-filling and for removing the deoxyribose 5'-phosphate (dRP) group formed as a result of the upstream DNA glycosylase and APE1 endonuclease activities^{1,2}. Because DNA is continuously damaged as a result of endogenous reactive oxygen and nitrogen species (RONs), aberrations in the BER pathway can be detrimental to the ability of cells to repair DNA, which can result in disease³. For example, approximately 30% of human cancers express mutations in pol β , many of which have been found to lead to cellular transformation⁴⁻¹². Mutations that alter pol β activity result in the generation and accumulation of toxic DNA intermediates, such as single strand breaks (SSBs) and double strand breaks (DSBs), which lead to genomic instability¹³. Alternatively, mutations that decrease the substrate specificity of pol β can lead to an increased mutational frequency, which is also detrimental to cells^{9, 14-20}. The mechanism by which a mutation can alter specificity of pol β is to either reduce discrimination at the level of polymerization rate (k_{pol}), known as chemistry or during binding and subsequent isomerization of the dNTP substrate, and this effect likely depends upon the location of the mutation within the pol β structure.

The goal of this study is to understand the contribution of the leaving group basicity to the catalytic mechanism and fidelity of the I260M prostate cancer-associated pol β variant. We previously demonstrated that the expression of I260M in non-transformed mammary epithelial cells induced focus formation and anchorage independent growth, which are hallmarks of cellular transformation²¹. Importantly, cellular transformation did not depend upon continuous expression of I260M, suggesting that it has a mutational basis. Follow-up studies showed that expression of I260M resulted in a mutation in peroxisome proliferator-activated protein γ 2 (PPARG2), which has been implicated as having tumor suppressor function. Therefore, mutations in PPARG2 caused by I260M pol β can drive cellular transformation⁶. Characterization of the mutational spectrum induced by I260M shows a ~3-fold increased mutational frequency as compared to wild-type (WT)⁹. Specifically, I260M exhibited a 6-fold increase in mutator activity in dipyrimidine sequences, due to

misalignment-mediated errors. Additionally, I260M had higher frequency of transversion mutations. Biochemical studies revealed that I260M pol β is a sequence context-dependent mutator variant, having very low fidelity, compared with WT pol β , within specific sequence contexts⁹. Importantly, I260M sequence context-dependent mutator activity resulted largely from defective discrimination at the level of dNTP binding to the protein.

Ile260 is located at the hydrophobic hinge region between the palm and fingers subdomains of pol β . The hinge region has been suggested to be important for dNTP binding and selectivity^{9, 18, 19, 22}. Alteration of Ile260 to Met was suggested to alter the hinge in a subtle manner, leading to destabilization of the ternary closed form of the polymerase. Molecular modeling studies suggest that the hinge region residue I260 is important for DNA positioning, which is important for forming the dNTP binding pocket. Consequently, it was suggested that a modification at position I260 may propagate structural alterations leading to increased ability of the enzyme to accommodate the incorrect dNTP¹⁸.

Here, we employ dNTP analogues that have been modified at the β - γ bridging group of the substrate dNTP (β , γ -CXY dNTP)^{23, 24}. These modifications change the basicity of the leaving group, represented by a change in the acid dissociation constant (pK_{a4}). This dNTP “toolkit”²⁴ has been previously used to show that the pre-steady state k_{pol} of WT pol β for incorporation of either correct or incorrect nucleotides is dependent on the pK_{a4} of the corresponding bisphosphonate leaving group. This dependence was attributed substantially to sensitivity of the TS free energy to internal charge stabilization modulated by the CXY electronegativity^{25–27}. Analysis of the results obtained with this tool-kit revealed that the TS formed during misincorporation is more sensitive to charge stabilization as compared to the TS formed during incorporation of correct nucleotide^{23, 25–27}. These effects are manifested in linear free energy relationship (LFER) plots of the logarithm of k_{pol} ($\log k_{pol}$) and the pK_{a4} of the leaving group, wherein a negative slope is indicative that the rate-determining step (RDS) is influenced by charge stabilization.

Most recently, we applied the dNTP tool-kit to study the pol β cancer-associated variant K289M, which has a mutator phenotype resulting from an increase in the frequency of inserting C opposite template C²⁷. This variant loses the dependence of $\log(k_{pol})$ on pK_{a4} observed with WT enzyme during correct incorporation but not during misincorporation, which pointed to a fundamental change in the RDS of K289M pol β relative to WT, with a shift to a step prior to chemistry²⁷. Moreover, it was found that several of the dNTP analogues tested improved the fidelity of the K289M cancer-associated variant. Consequently, these analogues could potentially be used to reduce mutational frequency caused by the K289M pol β cancer-associated variant.

Here, we examine the effect of the I260M mutation on correct and incorrect incorporation in the presence of tool-kit dNTP analogues. The results indicate that the dependence of I260M catalysis on the bisphosphonate leaving group pK_{a4} is similar to that of WT for both correct and incorrect incorporation. The I260M has improved fidelity with several dNTP analogues (CFCl, CBr₂, CHBr, CH₂), and our data suggest that this results from a tighter binding capacity to both correct and incorrect substrates.

MATERIALS AND METHODS

Chemistry

dTTP and dGTP were purchased from New England BioLabs and used as received. The dNTP analogues were synthesized and characterized by previously described methods^{23, 25, 28–30}: β,γ -CF₂ dTTP, β,γ -CFCI dTTP, β,γ -CCl₂ dTTP, β,γ -CHF dTTP, β,γ -CBr₂ dTTP, β,γ -CHCl dTTP, β,γ -CHBr dTTP, β,γ -CH₂ dTTP, β,γ -CF₂ dGTP, β,γ -CFCI dGTP, β,γ -CCl₂ dGTP, β,γ -CBr₂ dGTP, β,γ -CHF dGTP, β,γ -CHCl dGTP, β,γ -CHBr dGTP, β,γ -CH₂ dGTP, β,γ -CHCH₃ dGTP. The CXY compounds where X = Y were utilized as the $\approx$ 1:1 mixture of CXY diastereomers³¹.

Expression and Purification of DNA Polymerase β

WT and I260M pol β with an N-terminal 6x histidine tag were expressed in *Escherichia coli* BL21(DE3) and purified as previously described^{9, 27}.

DNA substrates

Oligonucleotides were purchased from the Keck Oligo Synthesis Resource at Yale University and were purified by polyacrylamide gel electrophoresis. The sequences used in this study are: primer – 5' GGCGAGGGCGCAACGCCGTACG, downstream – 5' CGGTTGCTATGGCCTCGAGAGA, and template – 5' CCGCTCCCGCGTTGCGGCATGCAGCCCAACGATACCGGAGCTCTCT, where A is the templating base in the gap between the primer and downstream oligonucleotides⁹. The duplex is referred to as the 45AC sequence (Figure 1A). The primer DNA sequence was phosphorylated at the 5' terminus using γ -³²P-ATP for detection and the downstream oligonucleotide was phosphorylated with ATP following manufacturer's instructions for T4 polynucleotide kinase (New England BioLabs). After phosphorylation, excess ATP was removed by passing the products through a microspin column. Primer, downstream and template oligonucleotides were mixed at a ratio of 1:1.6:1.3 and were allowed to anneal in annealing buffer containing 500 mM Tris-HCl, pH 8.0, and 2.5 M NaCl to generate a single base pair gapped DNA as previously described⁹.

Single Turnover Kinetics

250 nM pol β was pre-mixed with 50 nM labeled DNA (final concentrations), determined empirically for single turnover conditions³². Equal volumes of dNTP and pol β +DNA were mixed for specified times at 37 °C in 50 mM Tris pH 8.0, 10 mM 10 mM MgCl₂, 20 mM NaCl, 2 mM DTT and 10% glycerol. Reactions were quenched with 0.3 M EDTA using a KinTek rapid-quench flow apparatus. Manual quenching was performed for reactions longer than 120 seconds. Product DNA was separated on 20% PAGE containing 6M urea and phosphorescence emission was detected using a Storm 860 phosphoimager. ImageQuant software (GE Healthcare) was used to quantify the product and starting material, and fraction of product formed was then plotted as a function of time (t). Points were fitted to equation 1 using GraphPad Prism,

$$[\text{Product}] = A(1 - e^{-k_{\text{obs}}t}) \quad (\text{eq 1})$$

The observed rates (k_{obs}) were fitted to a hyperbolic equation as a function of dNTP concentration to identify k_{pol} and K_d , in accordance with equation 2.

$$k_{\text{obs}} = \frac{k_{\text{pol}}[\text{dNTP}]}{K_d + [\text{dNTP}]} \quad (\text{eq 2})$$

RESULTS

It has been previously shown that the I260M cancer-associated pol β variant has a reduced polymerization rate along with decreased fidelity resulting from poorer discrimination during ground state binding⁹. We have utilized a β,γ -CXY dTTP and β,γ -CXY dGTP analogue tool-kit to characterize correct and incorrect incorporation opposite template A to determine whether charge buildup at the TS during catalysis by I260M is altered. We also used this tool-kit to probe fidelity of the cancer variant. The primer-template sequence used in this study (Figure 1A) contains the sequence context in which strong mutator activity of I260M was demonstrated both *in vivo* and *in vitro*⁹.

The TS energy for I260M pol β catalysis is strongly dependent on leaving group charge stabilization during correct dTTP incorporation opposite template A

Tables 1 and 2 summarize the kinetics data for the WT and I260M in the presence of correct dTTP. For this study, we used 9 dTTP analogues with pCXYp pK_{a4} values ranging from 7.8 for β,γ -CF₂ to 10.5 for β,γ -CH₂, which were grouped into dihalogen substituted analogues and others. I260M incorporates the correct parent dTTP at about half the rate of the WT, where k_{pol} is 12.8 s⁻¹ for WT and 7.9 s⁻¹ for I260M. Alternatively, I260M binds the correct parent dTTP with 4-fold higher K_d (10.5 and 40.1 μM for WT and I260M, respectively). This results in reduced efficiency for the correct incorporation of dTTP by I260M ($0.2 \times 10^{-6} \mu\text{M}^{-1} \cdot \text{s}^{-1}$) as compared to WT ($1.2 \times 10^{-6} \mu\text{M}^{-1} \cdot \text{s}^{-1}$). Upon the addition of dTTP analogues, the efficiency of incorporation decreases for the WT, while the efficiency of I260M varied based on the leaving group.

The LFER correlation plot in Figure 1B shows that I260M has a dependency on negative charge stabilization in the leaving group similar to the WT. The dihalogenated LFER slope for the I260M is -1.01 ± 0.05 as compared to -1.22 ± 0.12 for the WT. Additionally, the slope of the line containing the “other” analogues is the same for WT and I260M (-0.81 ± 0.07 and -0.83 ± 0.06 , respectively). This suggests that the TS formed by both WT and I260M is sensitive to a similar extent to charge stabilization, and that the RDS for product formation is a chemical step.

The steeper dihalogenated slopes for WT and I260M suggest that there is more sensitivity to charge stabilization during formation of TS in the presence of the dihalogenated analogues, which has been shown previously for WT pol β ^{25–27}. This indicates that the dihalogenated

dTTP analogues are either exposed to or stimulating the formation of an altered binding pocket as compared to the other analogues.

TS formed during incorrect incorporation of dGTP opposite template A by I260M is dependent on charge stabilization

Figure 2 shows the LFER correlation plot for the misincorporation of dGTP opposite template A, and the kinetics data are summarized for WT and I260M in Tables 1 and 2, respectively. k_{pol} and K_d values for misincorporating the parent dGTP by WT and I260M are similar, thus, the efficiencies are similar. The introduction of dGTP analogues to WT pol β reduces the efficiency of dGTP insertion (Table 1). In contrast, the efficiency of I260M shows varied dependence on the incoming analogue (Table 2), the dihalogenated analogues exhibited the highest efficiencies, compared to the efficiency of the parent dGTP.

WT and I260M have similar LFER slopes. WT and I260M have slopes of -0.53 ± 0.09 and -0.47 ± 0.04 for the “other” lines and -0.76 ± 0.14 and -0.72 ± 0.13 for the dihalogenated lines, respectively. These results suggest that the TS formed during misincorporation is consistent with a chemical RDS that is similar for WT and I260M. The LFER slopes of the misincorporation of dGTP analogues, as compared to the accurate incorporation of dTTP analogues, indicate that the TS formed during misincorporation is less dependent on charge stabilization as compared to the TS formed during correct incorporation.

Misincorporation of dGTP opposite template A is less sensitive to charge stabilization as compared to correct dTTP incorporation

Previously, we have shown that misincorporation exhibits steeper slopes and increased sensitivity to charge dependence as compared to the corresponding correct incorporation. The pairs that have been previously studied include the misincorporation of dGTP opposite template T^{25, 26}, the misincorporation of dTTP opposite template G²⁶, and most recently, the misincorporation of dCTP opposite template C²⁷. These studies indicate that the TS occurring during misincorporation is highly sensitive to the charge stabilization effect created by the analogues as compared to the corresponding correct incorporation. The pair used in this study (dGTP opposite A) are both purines. The reduction in sensitivity to charge stabilization could result from increased dependence of TS formation on pre-chemical conformational changes.

A chemical rate determining step does not determine fidelity of I260M

Though both WT and I260M catalyze phosphodiester bond formation in a similar highly-charged TS, suggesting that the RDS is a chemical step, I260M has low fidelity as compared to WT (Table 3). In the presence of the parent dNTP, the fidelity of I260M is 5-fold lower than WT, however, both catalyze deoxynucleotide incorporation in a TS that has similar chemical characteristics, as identified by the slopes of both the correct and incorrect LFERs. This indicates that fidelity is not determined by the RDS of the reaction, as has been previously suggested^{33–35}.

Table 3 summarizes the fidelity of WT and I260M. WT pol β has the highest fidelity during incorporation of CF₂ and lowest fidelity with CH₂, however, fidelity does not significantly

change as compared to the parent dNTP (ratio ranging between 0.4–1.3 as compared to the parent). The fidelity of I260M changes modestly in the presence of some of the analogues, with two exceptions (CF₂ and CHF). The presence of CFCl substituted analogues improves I260M fidelity 11-fold as compared to the parent dNTP; alternatively, CBr₂ substituted analogues reduces fidelity 10-fold as compared to the parent dNTP. Compared to each other, the fidelity of I260M is much lower than that of the WT for all the analogues except for the CFCl substituted analogue.

Table 4 summarizes the ability of WT and I260M to discriminate against incorrect incorporation of the analogues at the level of ground state binding ($D K_d$). The presence of different analogues reduces discrimination by WT pol β at K_d as compared to the parent dNTP. This is caused by an increase in the correct K_d for most of the analogues. Discrimination by I260M is variable and dependent on the analogue. For example, the CFCl analogue increases discrimination by 14-fold resulting from a marked reduction in the correct K_d . In addition, CHBr and CH₂ increase discrimination by 4- and 5-fold, respectively.

The other mechanism by which pol β can discriminate against incorrect dNTP is at the level polymerization rate ($D k_{pol}$), shown in Table 5. There is a 2–5 fold increase in $D k_{pol}$ by WT relative to I260M as shown in the last column in Table 5. Meanwhile, the analogues have minimal effect on $D k_{pol}$ for I260M. The low fidelity of I260M, therefore, result from a loss in discrimination at the level of K_d not at the level of k_{pol} , more specifically, a loss in the ability to bind and isomerize the correct dTTP.

DISCUSSION

The hydrophobic hinge region located between the fingers and palm subdomain has been shown to be important for dNTP selectivity by allowing flexibility upon correct dNTP binding^{9, 18, 19, 22}. This region is important for the communication between the two subdomains. Therefore, it is not surprising that a mutation in this region alters the selectivity of pol β . Our data indicate that the TS during phosphodiester bond catalysis by I260M is dependent on the chemical properties of the incoming dNTP, however, fidelity of I260M is independent of the leaving group pK_{a4} .

Mutator activity of I260M results from lack of discrimination at ground state binding

The tool-kit used in this study contains dNTP analogues that are modified at the β - γ bridging group of the substrate dNTP. The purpose of this tool-kit is to understand the effect of charge stabilization on the TS formed during catalysis. Previous work has suggested that the TS has a highly-charged characteristic, where the 3'O⁻ of the primer terminus forms a partial phosphodiester bond with the α -P of the incoming nucleotide, meanwhile, the bond between α -P and its bridging oxygen is partially broken^{36, 37}. As a result, it is expected, and has been shown, that changing charge distribution at the β - γ bridge would affect the stability of this highly-charged TS. Consequently, this suggests that the formation of TS is limited by chemistry. Previous work from our laboratory on a cancer-associated pol β variant, K289M, showed that product formation is independent of charge stabilization for correct incorporation. This suggested that the TS state catalyzed by K289M is not the traditional

highly-charged TS and that it may be occurring during the formation of a pre-chemical state, possibly limited by a conformational change.

The I260M variant exhibits strong dependence on charge stabilization, indicating that the TS catalyzed by I260M during correct and incorrect incorporation is analogous to the proposed highly-charged TS catalyzed by WT. Therefore, the reduction in efficiency observed for I260M is not as a result of a change in the characteristic of TS. Our data indicate that the I260M variant exhibits decreased K_d for most of the correct nucleotides (Table 2), and reduced discrimination at the level of ground state binding, however, it is apparent that the nature of the leaving group has little effect on the ability of I260M to select a nucleotide.

The WT has the ability to modulate k_{pol} and K_d for correct and incorrect incorporation to minimize changes in fidelity in the presence of modified deoxynucleotides as compared to fidelity in the presence of the parent dNTP. The WT is able to overcompensate for the loss in discrimination at one level by improving discrimination at a different level. For example, a reduction in discrimination in the presence of CF₂ allows the WT enzyme to modulate the k_{pol} to increase discrimination; the resultant fidelity in the presence of CF₂ is similar to the parent dNTP. Therefore, the WT is able to coordinate between k_{pol} and K_d , to preserve maximal fidelity. In contrast, a mutation at Ile260 to Met may result in a deficiency in the ability to modulate discrimination between the binding and chemistry steps; therefore, a change in the chemical properties of leaving group will have an effect on fidelity. This loss of modulation between k_{pol} and K_d could result from an increase in the energy demand for the formation of any of the intermediates during the reaction mechanism.

Ile260 is located in the hydrophobic hinge region, between the dNTP binding subdomain (fingers) and the subdomain containing the active site components (palm). Crystal structures of pol β in the binary complex (in the absence of nucleotide) and in the ternary closed complex (in the presence of nucleotide) show that there is significant motion in the region upon dNTP binding³⁸. The hinge region is important for the flexibility of the fingers subdomain, which is necessary for closing the polymerase upon binding of the correct nucleotide and for alignment of the active site substituents for chemistry. Closing could become an energetically unfavorable event in the presence of a polar residue in place of the hydrophobic Ile at position 260. This will affect K_d and result in a misalignment of the active site, which reduces k_{pol} .

Molecular modeling of the energy minimized structure of I260M provides evidence of the structural effect of I260M on the nearby residues⁹. In this model, it is suggested the I260M causes an inefficient disruption of the salt bridge between D192 and R258 (Figure 3). In the WT enzyme, E295 and Y296 at the hinge interact with R258 in the closed structure to draw away R258 from interacting with D192. In the presence of a polar group in the hydrophobic hinge region, for example I260M, E295 may not efficiently interact with R258, which allows R258 to keep its salt bridge with D192. D192 is one of the key residues that coordinates the active site metal, therefore, it is important to have a free D192 for chemistry⁹.

Ile260 lines the outside of the hinge region, along with Thr196 and Tyr265³⁸. It was proposed that changes in this region would have long-range effects on the dNTP binding

pocket¹⁸. Several cancer-associated variants were found to be located in this region such as I174S and Y265C^{19, 39}. The I174S cancer-associated variant, for example, has been shown to cause a reduction in discrimination at the level of K_d ¹⁹. Additional hinge region mutator mutants were also found to lose discrimination at K_d , including Y265H, F272L, I260Q^{40–42}.

It has been shown by molecular modeling that the hydrophobicity of this region is essential for selectivity and that maintaining this characteristic strongly affects accuracy¹⁸. Therefore, it is not surprising that introducing some polarity from the sulfur atom in Met as compared to the hydrophobic Ile would disrupt the arrangement in the hinge region, which may translate into a change in accuracy.

Fidelity of I260M is improved in the presence of the CFCI modified dNTP

In addition to providing information on the TS and the dependence of the TS on negative charge stabilization in the leaving group, the analogues can be used as a tool to modify fidelity. In the presence of the CFCI modified dNTP, the fidelity of I260M is improved relative to WT (2-fold increase) and relative to the parent dNTP (11-fold increase). This may be due to an increase in the binding affinity for the analogue relative to the parent (3.1 μ M for CFCI-dTTP and 40.1 μ M for the parent dTTP). This specific analogue can be used to reduce the frequency of misinsertions because I260M binds more tightly to the correct CFCI analogue suggesting that ground state binding energy differences propagate, at least partially, in the TS.

CONCLUSIONS

This work provides strong evidence that the hinge region is important for selectivity of pol β at the level of ground state nucleotide binding and that modifications at Ile260 do not affect the formation of the TS. We also show that changes in the fidelity of I260M in the presence of the analogues are nonspecific and may result from a lack of coordination between the fingers and palm subdomains; however, I260M fidelity improved drastically in the presence of the CFCI analogue. Finally, this work provides additional evidence that the TS energy is modulated by the nucleotide pair.

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ABBREVIATIONS

BER	base excision repair
dRP	deoxyribose 5'-phosphate
dNTP	deoxynucleoside 5'-triphosphate
K_d	dNTP dissociation constant of polymerase β

k_{pol}	polymerization rate of gapped DNA
LFER	linear free energy relationship
RDS	rate-determining step
RONs	reactive oxygen and nitrogen species
TS	transition state
WT	wild-type

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A

Template-Primer sequence (45AC):

5' GGCGAGGGCGCAACGCCGTACG CGGTTGCTATGGCCTCGAGAGA 3'
 3' CCGCTCCCGCGTTGCGGCATGC AGCCAACGATACCGGAGCTCTCT 5'

B**Correct LFER**

Figure 1. Linear free energy relationships for the correct incorporation of dTTP opposite template A

(A) 45AC Template-Primer sequence was used in this study, which is the sequence that exhibited lowest fidelity with I260M. (B) $\log(k_{\text{pol}})$ vs leaving group pK_{a4} for the correct incorporation of dTTP analogues opposite template A by WT and I260M pol β . Points were fitted with the equation of a line to generate slopes for WT other (Blue, -0.81 ± 0.07), WT dihalogenated (Red, -1.22 ± 0.12), I260M other (Green, -0.83 ± 0.06), and I260M dihalogenated (Purple, -1.01 ± 0.05).

Figure 2. Linear free energy relationships for the incorrect incorporation of dGTP opposite template A

$\log(k_{\text{pol}})$ vs leaving group pK_{a4} for the incorrect incorporation of dGTP analogues opposite template A by WT and I260M pol β . Points were fitted with the equation of a line to generate slopes for WT other (Blue, -0.53 ± 0.09), WT dihalogenated (Red, -0.76 ± 0.14), I260M other (Green, -0.47 ± 0.04), and I260M dihalogenated (Purple, -0.72 ± 0.13).

Figure 3. Effect of I260 on active site residues

The open binary structure is shown in red and the ternary closed conformation is in blue. The salt bridge between D192 and R285 that occurs in the open binary conformation needs to be efficiently disrupted in order to free D192 to coordinate with the metal and the incoming nucleotide. This disruption occurs when E295 interacts with R285. The I260M mutation is expected to disrupt the hinge region, which impairs E295 from interacting with R285. Residues in the open position are shown in dark red and in the closed conformation are shown in light blue. The incoming dNTP is shown in yellow and the β,γ -bridging group is shown in brown.

Table 1

Summary of WT Kinetic Data from Single Turnover Activity

M-N ^a	X-	pK _{ad} ^b	k _{pol} (s ⁻¹)	K _d (μM)	Efficiency (μM ⁻¹ s ⁻¹) ^c
A-T	CF ₂	7.8	14.6 ± 1.80	34.2 ± 8.30	0.43 ± 0.12
A-T	CFCI	8.4	3.80 ± 0.30	27.5 ± 6.10	0.14 ± 0.03
A-T	CCl ₂	8.8	0.66 ± 0.09	44.7 ± 20.2	0.015 ± 0.007
A-T	O	8.9	12.8 ± 0.60	10.5 ± 1.10	1.22 ± 0.14
A-T	CHF	9.0	18.3 ± 1.10	83.6 ± 9.20	0.22 ± 0.03
A-T	CBF ₂	9.3	0.25 ± 0.01	71.7 ± 10.8	0.004 ± 0.001
A-T	CHCl	9.5	5.90 ± 0.50	30.3 ± 7.30	0.19 ± 0.05
A-T	CHBr	9.9	2.80 ± 0.10	9.80 ± 2.10	0.29 ± 0.06
A-T	CH ₂	10.5	0.80 ± 0.10	6.70 ± 1.80	0.12 ± 0.03
A-G	CF ₂	7.8	6.0E-03 ± 6.0E-04	1009 ± 197	5.9E-06 ± 1.3E-06
A-G	CFCI	8.4	1.8E-03 ± 3.0E-04	833 ± 345	2.2E-06 ± 9.6E-07
A-G	CCl ₂	8.8	6.0E-04 ± 1.0E-04	1403 ± 482	4.3E-07 ± 1.6E-07
A-G	O	8.9	1.4E-02 ± 2.0E-03	626 ± 220	2.2E-05 ± 8.5E-06
A-G	CHF	9.0	6.0E-03 ± 9.0E-04	786 ± 293	7.6E-06 ± 3.1E-06
A-G	CBF ₂	9.3	5.0E-04 ± 1.0E-04	67 ± 17	7.5E-06 ± 2.4E-06
A-G	CHCl	9.5	7.0E-03 ± 2.0E-03	1000 ± 500	7.0E-06 ± 4.0E-06
A-G	CHBr	9.9	2.6E-03 ± 3.0E-04	205 ± 79	1.3E-05 ± 5.1E-06
A-G	CH ₂	10.5	3.0E-03 ± 2.0E-04	1161 ± 189	2.6E-06 ± 4.5E-07
A-G	CHCH ₃	11.6	3.0E-04 ± 4.0E-05	57 ± 47	5.3E-06 ± 4.4E-06

^a M-N is the template-incoming dNTP and X- is the β,γ-bridging group.

^b pK_{ad} values from Sucato et al., 2008.

^c Efficiency is k_{pol}/K_d.

Table 2

Summary of I260M Kinetic Data from Single Turnover Activity

M-N ^a	X-	pK _{ad} ^b	k _{pol} (s ⁻¹)	K _d (μM)	Efficiency (μM ⁻¹ s ⁻¹) ^c
A-T	CF ₂	7.8	5.80 ± 1.40	67.2 ± 25.3	0.09 ± 0.04
A-T	CFCI	8.4	1.30 ± 0.10	3.10 ± 0.50	0.42 ± 0.07
A-T	CCl ₂	8.8	0.66 ± 0.09	20.9 ± 9.80	0.032 ± 0.015
A-T	O	8.9	7.90 ± 1.30	40.1 ± 15.5	0.20 ± 0.08
A-T	CHF	9.0	5.60 ± 1.70	86.4 ± 46.5	0.06 ± 0.04
A-T	CBF ₂	9.3	0.19 ± 0.01	43.9 ± 10.5	4.3E-03 ± 1.1E-03
A-T	CHCl	9.5	3.20 ± 0.30	55.5 ± 13.6	0.06 ± 0.02
A-T	CHBr	9.9	1.30 ± 0.10	6.20 ± 0.50	0.21 ± 0.02
A-T	CH ₂	10.5	0.30 ± 0.01	5.00 ± 1.50	0.06 ± 0.02
A-G	CF ₂	7.8	1.3E-02 ± 2.0E-03	1289 ± 397	1.0E-05 ± 3.5E-06
A-G	CFCI	8.4	3.0E-03 ± 3.0E-04	886 ± 220	3.4E-06 ± 9.1E-07
A-G	CCl ₂	8.8	3.0E-03 ± 2.0E-04	277 ± 70	1.1E-05 ± 2.8E-06
A-G	O	8.9	1.4E-02 ± 3.0E-03	795 ± 373	1.8E-05 ± 9.1E-06
A-G	CHF	9.0	7.0E-03 ± 1.0E-03	1100 ± 328	6.4E-06 ± 2.1E-06
A-G	CBF ₂	9.3	9.0E-04 ± 1.0E-04	177 ± 87	5.1E-06 ± 2.6E-06
A-G	CHCl	9.5	5.8E-03 ± 1.0E-03	681 ± 304	8.5E-06 ± 4.1E-06
A-G	CHBr	9.9	4.0E-03 ± 7.0E-04	454 ± 179	8.8E-06 ± 3.8E-06
A-G	CH ₂	10.5	1.6E-03 ± 2.0E-04	515 ± 194	3.1E-06 ± 1.2E-06
A-G	CHCH ₃	11.6	6.0E-04 ± 8.0E-05	236 ± 107	2.5E-06 ± 1.2E-06

^a M-N is the template-incoming dNTP and X- is the β,γ-bridging group.

^b pK_{ad} values from Sucato et al., 2008.

^c Efficiency is k_{pol}/K_d.

Table 3

Fidelity of WT and I260M

Enzyme	-X-	pK _a ^a	F analogue ^a	F (analogue/parent) ^b	I260M/WT analogue ^c	I260M/WT (analogue/parent) ^d
WT						
	CF ₂	7.8	72357	1.3		
	CFCI	8.4	63637	1.1		
	CCl ₂	8.8	34885	0.6		
	O	8.9	55412	1.0		
	CHF	9.0	28804	0.5		
	CBr ₂	9.3	534.0	0.0		
	CHCl	9.5	27818	0.5		
	CHBr	9.9	21979	0.4		
	CH ₂	10.5	44777	0.8		
I260M						
	CF ₂	7.8	9001.0	0.8	0.1	0.6
	CFCI	8.4	123530	11.1	1.9	9.7
	CCl ₂	8.8	2910.0	0.3	0.1	0.4
	O	8.9	11112	1.0	0.2	1.0
	CHF	9.0	9376.0	0.8	0.3	1.6
	CBr ₂	9.3	844.0	0.1	1.6	7.9
	CHCl	9.5	7060	0.6	0.3	1.3
	CHBr	9.9	23865	2.1	1.1	5.4
	CH ₂	10.5	19356	1.7	0.4	2.2

^aFidelity, F, is calculated from [efficiency(correct)+efficiency(correct)]/efficiency(incorrect)^bF analogue/F parent^c[F I260M(analogue)]/[F WT (analogue)]^d[F I260M(analogue/parent)]/[F WT (analogue/parent)]

Table 4Discrimination at K_d by WT and I260M

Enzyme	-X-	pK _a	D K_d (t/c) analogue ^a	D K_d (analogue/parent) ^b	D K_d analogue I260M/WT ^c
WT	CF ₂	7.8	29.5	0.5	
	CFCI	8.4	30.3	0.5	
	CCl ₂	8.8	31.4	0.5	
	O	8.9	59.6	1.0	
	CHF	9.0	9.4	0.2	
	CBr ₂	9.3	0.9	0.0	
	CHCl	9.5	33.0	0.6	
	CHBr	9.9	20.9	0.4	
	CH ₂	10.5	173.3	2.9	
I260M	CF ₂	7.8	19.2	1.0	0.7
	CFCI	8.4	285.8	14.4	9.4
	CCl ₂	8.8	13.3	0.7	0.4
	O	8.9	19.8	1.0	0.3
	CHF	9.0	12.7	0.6	1.4
	CBr ₂	9.3	4.0	0.2	4.3
	CHCl	9.5	12.3	0.6	0.4
	CHBr	9.9	73.2	3.7	3.5
	CH ₂	10.5	103.0	5.2	0.6

^aDiscrimination, D, at K_d is calculated from K_d (incorrect)/ K_d (correct)^bD K_d analogue/ D K_d parent^cD K_d I260M analogue/ D K_d WT analogue

Table 5

Discrimination at k_{pol} by WT and I260M

Enzyme	X-	pK _a	D k_{pol} (cf) analogue ^a	D k_{pol} (analogue/parent) ^b	D k_{pol} analogue WT/I260M ^c
WT	CF ₂	7.8	2433.3	2.7	5.5
	CFCI	8.4	2111.1	2.3	4.9
	CCl ₂	8.8	1100.0	1.2	5.0
	O	8.9	914.3	1.0	1.6
	CHF	9.0	3050.0	3.3	3.8
	CBr ₂	9.3	500.0	0.5	2.4
	CHCl	9.5	842.9	0.9	1.5
	CHBr	9.9	1076.9	1.2	3.3
	CH ₂	10.5	260.0	0.3	1.3
I260M	CF ₂	7.8	446.2	0.8	
	CFCI	8.4	433.3	0.8	
	CCl ₂	8.8	220.0	0.4	
	O	8.9	564.3	1.0	
	CHF	9.0	800.0	1.4	
	CBr ₂	9.3	211.1	0.4	
	CHCl	9.5	551.7	1.0	
	CHBr	9.9	325.0	0.6	
	CH ₂	10.5	200.0	0.4	

^aDiscrimination, D, at k_{pol} is calculated from $k_{\text{pol}}(\text{correct})/k_{\text{pol}}(\text{incorrect})$ ^bD k_{pol} analogue/ D k_{pol} parent^cD k_{pol} WT analogue/ D k_{pol} I260M analogue