Published in final edited form as:

Biochemistry. 2017 April 18; 56(15): 2096–2105. doi:10.1021/acs.biochem.6b01230.

A Change in the Rate-Determining Step of Polymerization by the K289M DNA Polymerase β Cancer-Associated Variant

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Abstract

K289M is a variant of DNA polymerase β (pol β) that has previously been identified in colorectal cancer. The expression of this variant leads to a 16-fold increase in mutation frequency at a specific site *in vivo* and a reduction in fidelity *in vitro* in a sequence context-specific manner. Previous work shows that this reduction in fidelity results from a decreased level of discrimination against incorrect nucleotide incorporation at the level of polymerization. To probe the transition state of the K289M mutator variant of pol β , single-turnover kinetic experiments were performed using β , γ -CXY dGTP analogues with a wide range of leaving group monoacid dissociation constants (pK_{a4}), including a corresponding set of novel β , γ -CXY dCTP analogues. Surprisingly, we found that the values of the log of the catalytic rate constant (k_{pol}) for correct insertion by K289M, in contrast to those of wild-type pol β , do not decrease with increased leaving group pK_{a4} for analogues with pK_{a4} values of <11 . This suggests that one of the relative rate constants differs for the K289M reaction in comparison to that of the wild type (WT). However, a plot of $\log(k_{pol})$ values for incorrect insertion by K289M versus pK_{a4} reveals a linear correlation with a negative slope, in this respect resembling k_{pol} values for misincorporation by the WT enzyme. We also show that some of these analogues improve the fidelity of K289M. Taken together, our data show that Lys289 critically influences the catalytic pathway of pol β .

Graphical abstract

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Supporting Information: The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b01230. Synthetic details and NMR and MS spectral data for 2a-f (PDF)

Notes: The authors declare no competing financial interest.

DNA polymerase β (pol β) is essential for the repair of damaged DNA by base excision repair (BER). BER prevents cells from propagating DNA damage caused by exposure to endogenous and exogenous sources. Aberrations in this pathway have proven to be detrimental and are associated with human diseases, saliently including cancer.¹ Pol β has been shown to be mutated in a variety of human tumors.^{2,3} Several of the cancer-associated pol β variants possess aberrant function *in vitro*, including slowed or more error-prone DNA polymerase activity.^{4–8} Importantly, expression of these pol β variants in cells induces genomic instability and cellular transformation.^{2,6,7,9,10} Thus, understanding the basic mechanisms of catalysis by pol β , including the fidelity of DNA synthesis, will provide important insights into carcinogenesis.

Pol β is a member of the X-family of DNA polymerases.¹¹ It is a small protein with a molecular weight of 39 kDa and has two functional domains, the 8 kDa lyase and 31 kDa polymerase domains. The 8 kDa lyase domain catalyzes the β -elimination of the remaining 5'-deoxyribose phosphate (dRP) group from the preceding phosphodiesterase reaction.¹² The polymerase domain catalyzes a three-metal ion-dependent mechanism involving nucleophilic attack by the 3'-OH of the gapped DNA on P α of the incoming dNTP to form a phosphodiester bond.^{13,14} This reaction results in the formation of an extended DNA product with the release of inorganic pyrophosphate (PP_i). The polymerase domain of pol β is organized into three subdomains resembling a right-handed structure: the thumb subdomain (residues 88–147) binds to DNA, the palm subdomain (residues 148–261) contains components of the active site, and the fingers subdomain (residues 262–335) binds the incoming dNTP.^{15,16}

We have previously shown that amino acid residues distant from the active site of pol β influence fidelity in the ground state.¹⁷ Using two different genetic screens,^{18,19} we identified several mutator variants of pol β , each with single-amino acid alterations in residues distant from the active site of the protein. Subsequent biochemical characterization of these variants demonstrated that they exhibited fidelity lower than that of the WT enzyme, mostly because of a lack of substrate discrimination during ground state binding.^{20–29} We have also shown that variants of pol β with low fidelity found in human tumors result from single-amino acid alterations located at positions far from the active site.^{2,4,10,17,30,31} Together, these studies suggest that the fidelity of pol β is affected by amino acids distant from the active site of the protein that may not participate directly in catalysis but may influence substrate choice by affecting the conformation of the enzyme. In support of this, we have recently used fluorescence resonance energy transfer (FRET) to monitor precatalytic conformational movements of pol β and to show that the fingers domain of pol β does not close in a stable manner in the presence of the incorrect dNTP substrate. In addition, recent NMR spectroscopic characterization of pol β suggests that this enzyme closes stably in the presence of correct dNTP, but not in the presence of a mismatch. It has also been suggested that prechemistry conformational changes govern fidelity in other polymerases such as HIV RT and T7 polymerase. Taken together, these studies suggest that specific amino acid residues distant from the active site of pol β promote stable precatalytic conformational changes that influence fidelity, supporting an induced-fit mechanism.

Pol β does not contain a proofreading function, which reduces its overall fidelity. It is estimated that pol β misinserts nucleotides at a frequency of 1 in ~5000.³⁹ To increase specificity and fidelity, pol β utilizes multiple kinetic steps to govern substrate selection.^{32,40–42} Previous evidence indicates that, for pol β , chemistry is normally rate-determining for both correct and incorrect nucleotides, though the activation energy barrier for the forward reaction of the incorrect dNTP is much higher.^{40,42–46} It has been suggested that the difference in energy results from a distorted active site in the presence of a mismatch.^{33,44} Reverse fingers closing has also been shown to be an important determinant of fidelity for HIV-RT as the enzyme prepares for chemistry.^{34,35}

It has been proposed that pol β employs a number of mechanisms to select the correct dNTP substrate, including ground state binding, prechemistry conformational change(s), and transition state (TS) chemistry.^{32,40,42} Conformational changes occur upon binding of the correct dNTP, which result in closing of the fingers subdomain and other smaller movements that align the active site for an inline nucleophilic attack with the incoming dNTP, as shown by structural and FRET studies.^{13–15,32,33} This then commits the enzyme to chemistry particularly if the reverse fingers opening is a slow reaction compared to the rate of chemistry.^{34,35} The chemical reaction requires deprotonation of the 3'-OH to assist with the nucleophilic attack of the 3'-oxygen on P α of the bound dNTP. Ultimately, the latter bond is broken, and PP $_i$ is eliminated as the leaving group.^{13,14,47}

In accordance with TS theory, the TS is defined as the highest-free energy state along the reaction coordinate, which constitutes the rate-determining step (RDS).^{48,49} A tool kit consisting of dGTP analogues modified by installation of β,γ -CXY bridging groups was introduced to study leaving group effects on the TS of pol β during the incorporation of correct and incorrect nucleotides.^{46,50,51} Via variation of the X and Y substituents, the pK $_{a4}$ of the corresponding pCXYp methylene (bisphosphonate) leaving group can be modulated to probe the dependence of k_{pol} on the basicity of the leaving group. Thus, more electronegative substituents on the bridging β -methylene decrease pK $_{a4}$, corresponding to a more stable leaving group anion, producing a leaving group effect and increasing k_{pol} .^{50,51} This effect can be quantitated by fitting the experimental k_{pol} and pK $_{a4}$ data to the Brønsted equation,^{46,50–52} which postulates a linear free energy relationship (LFER)⁴⁸ between the logarithm of the rate constant (log k) and the logarithm of the last acid dissociation constant (pK $_a$) for the leaving group, in accordance with eq 1^{48,49}

$$\log k_{\text{pol}} = \beta \times \text{p}K_{a4} + C \quad (1)$$

where k_{pol} is the experimental reaction rate constant and coefficient β is related to the sensitivity of k_{pol} to a change in leaving group basicity; if a more basic leaving group increases the TA G , $\beta < 0$. The intercept, C , is a constant.

Previous single-turnover kinetic studies using dGTP and dTTP bisphosphonate analogues demonstrated that their polymerase-catalyzed turnover reaction rate constants for both correct and incorrect base pairing to the DNA template exhibit well-defined linear Brønsted

correlations with negative slopes,^{50,51} consistent with a chemical RDS in both cases. These studies also showed that the absolute value of β for incorrect base pairs was typically larger than those for the correct base pairs, showing a greater demand for charge stabilization in the mispair mechanism.^{50,51}

K289M is a colon cancer-associated variant of pol β , wherein a lysine residue, located in the fingers subdomain at the end of helix N, is mutated to a methionine.¹⁷ Helix N is important for closing of the fingers subdomain upon binding of the correct dNTP.¹³ The K289M variant exhibits a 16-fold increase in spontaneous mutation frequency *in vivo* within a specific sequence context, identical to a frequently mutated site within the adenomatous polyposis coli (APC) gene.¹⁷ When tested *in vitro*, this variant exhibits reduced fidelity by misincorporating dCTP opposite template C in a sequence context-specific manner, as a result of a reduced level of discrimination at the level of polymerization.¹⁷

In this study, we investigated the base-dependent kinetic mechanism of DNA synthesis catalyzed by the cancer-associated K289M variant of pol β , using a set of CXY dNTP probes that includes novel dCTP as well as dGTP analogues to compare both pyrimidine substrate and corresponding purine substrate base recognition effects on catalysis. Surprisingly, in contrast to the results for WT pol β , k_{pol} for the K289M mutant enzyme is not dependent on the CXY dGTP leaving group for correct incorporation opposite template C ($\beta \sim 0$) but is dependent for the misincorporation of dCTP opposite template C ($\beta < 0$). The results provide evidence that even though it is remote from the active site in the ground state conformation, Lys289 plays a critical role in the catalysis of the correct nucleotidyl transfer reaction for dGTP, playing an important role in accurate DNA synthesis.

Materials and Methods

Synthesis of dNTP Analogues

Cytidine 5'-monophosphate was purchased from Chem Impex International. The methylene bisphosphonic acids [in the form of their anhydrous tris (tributylammonium) salts] and cytidine 5'-monophosphate morpholidate were prepared according to published procedures.^{53–55} All other reagents were purchased from commercial sources and used as obtained. Preparation of the dGTP β, γ -CXY analogues 1a–i was reported previously.^{46,50,51,56,57} The corresponding β, γ -CXY analogues of dCTP, 2a–g, were prepared analogously. Thus, to cytidine 5'-monophosphate morpholidate [1 equiv in 2 mL of anhydrous dimethyl sulfoxide (DMSO)] was added the appropriate member of the group of methylenebisphosphonates 3a–i [tris(tributylammonium) salt, 3 equiv in 3 mL of dry DMSO]. After 48 h, volatiles were removed under reduced pressure. The residue was dissolved in 0.5 N triethylammonium bicarbonate (TEAB) buffer (pH 7–8) and subjected to dualpass preparative high-performance liquid chromatography (HPLC) (Varian ProStar, Shimadzu SPD-10A UV detector/0.5 mm path, 267 nm). For SAX: Macherey-Nagel 21.4 mm $\times$ 250 mm SP15/25 Nucleogel column, eluted with 5% acetonitrile in water and 0.5 M TEAB (pH 7.5), from 0 to 40% over 10 min, 40% for 6 min, and from 40 to 100% over 9 min, 8 mL/min. For RP: 21.4 mm $\times$ 250 mm Microsorb 100-5 C18 column, eluted isocratically with 0.1 M TEAB buffer (pH 7.5) and 8.5% acetonitrile, 8 mL/min. The purified nucleotide analogue product was characterized by ¹H, ¹⁹F, and ³¹P NMR (Varian

400), MS (ESI Finnigan LCQ Deca XP Max in negative ion mode) (Figures S1–S21) and by analytical HPLC.

Expression and Purification of DNA Polymerase β

WT and K289M pol β with an N-terminal six-histidine tag were expressed in *Escherichia coli* BL21(DE3) and purified as previously described.¹⁷ Briefly, cells were collected and lysed by sonication. The lysate was loaded onto a nickel-charged chelating column, and protein was eluted with 250 mM imidazole in buffer A [50 mM Tris (pH 8.0) and 100 mM NaCl] after column washes with buffer A using fast protein liquid chromatography. The eluent was loaded onto an SP column, and pol β was eluted with 1 M NaCl in buffer B [50 mM Tris (pH 8.0), 1 mM EDTA, and 10% glycerol]. The protein concentration was determined by A_{280} using $21200 \text{ M}^{-1} \text{ cm}^{-1}$ as the extinction coefficient.

DNA Substrates

Oligonucleotides were purchased from the Keck Oligo Synthesis Resource at Yale University and were purified by polyacrylamide gel electrophoresis. The following sequences were used in this study: primer (5' GAACTCCAT-ATGGATTT), downstream (5' TTCAGAACGCTCGGT-TGC), and template (5' GCAACCGAGCGTTCTGAACAAA-TCCATATGGAGTTC, where C is the templating base in the gap between the primer and downstream oligonucleotides).¹⁷ The duplex was termed the CL-CP-CG sequence. 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 the 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 in a 1:1.6:1.3 ratio 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 described.¹⁷

Single-Turnover Kinetics

Pol β (750 nM) was premixed with 50 nM labeled DNA (final concentration), a ratio that was empirically determined for single-turnover conditions.⁵⁸ Reactions were initiated by mixing with equal volumes of dNTP and 10 mM Mg^{2+} for specified times at 37 °C in 50 mM Tris (pH 8.0), 20 mM NaCl, 2 mM DTT, and 10% glycerol and then quenched with 0.3 M EDTA using a KinTek rapid quench-flow apparatus. Reactions that took >120 s were quenched manually. The correct dGTP analogues were titrated in a concentration range of 0.05–250 μM . Incorrect dCTP analogues were titrated in a concentration range of 25–2000 μM . Reaction products were separated via 20% polyacrylamide gel electrophoresis containing 6 M urea. Dehydrated gels were exposed to a phosphor screen, and the signal was detected by phosphorescence emission. The intensities of the product and starting material bands were quantified using ImageQuant (GE Healthcare); the fraction of product formed was plotted as a function of time (t). Points were fitted to a single-exponential equation (eq 2) using GraphPad Prism to obtain an observed rate (k_{obs}) for each of the dNTP concentrations

$$[\text{product}] = A \left(1 - e^{-k_{\text{obs}} t} \right) \quad (2)$$

The k_{obs} rates were plotted as a function of dNTP concentration and were fit to a hyperbolic equation to identify k_{pol} and K_d in accordance with eq 3.

$$k_{\text{obs}} = \frac{k_{\text{pol}} [\text{dNTP}]}{K_d + [\text{dNTP}]} \quad (3)$$

Reactions were performed in triplicate and are reported as means $\pm$ the standard deviation.

Results

Using the β, γ -CXY dGTP analogue suite, we previously found that the rate constant for pol β -catalyzed nucleotide insertion into single-nucleotide gapped DNA decreased with increasing leaving group basicity, suggesting that a charge-altering chemical process is involved in the RDS.^{46,50,51} In the study reported here, our goal was to determine if the rates of the K289M pol β sequence context-specific mutator variant also decreased with increasing leaving group basicity.¹⁷

Synthesis of dNTP Analogues

β, γ -CXY dGTP analogues **1a–j** (Figure 1) have been reported previously.^{46,50,51,56,57}

The corresponding β, γ -CXY analogues of dCTP have not been previously described but were successfully prepared by similar methods. Thus, the morpholidate of cytidine 5'-monophosphate **4**^{53,54} was reacted with the appropriate methylene bisphosphonate **3** and product dCTP analogue **2** obtained in high purity (>99%) by preparative HPLC. Compounds **1b–d**, **1g**, **2a**, **2c**, **2e**, and **2f** were prepared and utilized as ~1:1 CXY (X = Y) diastereomer mixtures.⁵⁵

Rates for Correct Incorporation by K289M Are Independent of the Leaving Group

We initially measured the k_{pol} and K_d values of WT and K289M for incorporation of the correct dGTP within the mutator sequence context. Representative kinetic traces and the saturation binding plot for the parent dGTP are shown in Figure S22. WT pol β binds tightly to the parent dGTP ($K_d = 2.3 \pm 0.3 \mu\text{M}$) and has rapid turnover activity ($k_{\text{pol}} = 27.6 \pm 1.2 \text{ s}^{-1}$). A significant catalytic phenotype of the K289M cancer variant is the slow rate of polymerization of the correct dGTP nucleotide as compared to that of WT ($k_{\text{pol}} = 0.9 \pm 0.1 \text{ s}^{-1}$; ~30-fold reduction), although it binds tightly to dGTP ($K_d = 1.3 \pm 0.3 \mu\text{M}$).

Next, we measured k_{pol} and K_d for correct incorporation of a series of dGTP analogues opposite template C by WT and the K289M variant. Figure 2 shows representative kinetic traces (A and C) and saturation plots (B and D) for the incorporation of a β, γ -CH₂-dGTP analogue opposite template C by WT and K289M pol β , respectively.

Tables 1 (WT) and 2 (K289M) report k_{pol} and K_{d} values for each of the analogues. The tested analogues are grouped as previously reported:^{25,30,31} analogues that contain dihalogenated substituents as one group termed the dihalogenated dNTPs and the remaining analogues containing methylated, monohalogenated, and parent dNTPs grouped together and termed “the other analogues”.

$\log(k_{\text{pol}})$ and $\text{p}K_{\text{a4}}$ for the other analogues correlate in a linear fashion, as shown in Figure 3A. WT pol β exhibits a strong dependence of $\log(k_{\text{pol}})$ on the $\text{p}K_{\text{a4}}$ of the bisphosphonate leaving groups, with a slope of -0.4 (Figure 3A, blue line), whereas β is close to zero for the K289M variant [-0.046 (Figure 3A, red line)]. This suggests that one of the relative rate constants of the reaction mechanism may differ for the K289M cancer-associated variant compared to that of WT pol β .

The β,γ -CHF bisphosphonate leaving group has a $\text{p}K_{\text{a4}}$ close to that of pyrophosphate from the natural parent dGTP,⁵⁵ and although the binding affinity decreases 3-fold, k_{pol} is similar to that of the parent dGTP. The β,γ -CHCl bisphosphonate has a $\text{p}K_{\text{a4}}$ of 9.5; in addition to a 3-fold decrease in binding affinity, k_{pol} decreases 1.5-fold. The β,γ -CH₂ bisphosphonate has a high $\text{p}K_{\text{a4}}$ of 10.5, compared to a value of 8.9 for pyrophosphate. This is reflected in the corresponding k_{pol} of 10.1 s^{-1} , which is 3-fold lower than that of the parent dGTP.

Similar results were obtained with the dihalogenated compounds, which is shown in Figure 3B (blue line, WT; red line, K289M). This relationship shows that the WT activity follows a strong negative dependence on $\text{p}K_{\text{a4}}$ (β is -0.63). The catalytic rate decreases from 44 s^{-1} for the β,γ -CF₂ dGTP, which has a $\text{p}K_{\text{a4}}$ of 7.8, to 3.7 s^{-1} for the β,γ -CBr₂, which has a $\text{p}K_{\text{a4}}$ of 9.3 (Table 1). Strikingly, for K289M, the dependence of k_{pol} on $\text{p}K_{\text{a4}}$ is less prominent: the rate decreases from 0.8 s^{-1} for the β,γ -CF₂ dGTP to 0.3 s^{-1} for the β,γ -CBr₂ (Table 2), and the slope of the Brønsted correlation for K289M is -0.25 for the dihalogenated analogues (Figure 3B, red line). In combination, our results suggest that the K289M variant has a TS that is less dependent on charge stabilization than WT pol β is. We speculate that for K289M there may be a change in the equilibrium constant for one or more of the conformational changes that precede chemistry.³²

The Correct LFERs Break at High $\text{p}K_{\text{a4}}$ Values

The WT enzyme is strongly dependent on $\text{p}K_{\text{a4}}$, and the mutant is independent of $\text{p}K_{\text{a4}}$ up to values of 10.5. However, the Brønsted relationship for both WT and K289M pol β displays a break at high $\text{p}K_{\text{a4}}$ (Figure 3A). This is likely to represent a change in the TS structure for the WT. The rate of polymerization by WT becomes more dependent on the $\text{p}K_{\text{a4}}$, indicating that the TS is less stable at high $\text{p}K_{\text{a4}}$. For the K289M variant, this break indicates a change in the RDS at $\text{p}K_{\text{a4}} > 10.5$ from a prechemistry step where a leaving group effect is not observed to a chemistry step where the basicity of the leaving group moiety influences the rate. A break of this nature, for WT or K289M, has not been reported previously.

K289M and WT Exhibit a Similar Dependence on the $\text{p}K_{\text{a4}}$ of the Leaving Group for Incorrect Incorporation

The LFER for incorrect dCTP incorporation for each of the enzymes is linear and is shown in Figure 4.

The WT and K289M enzymes are similarly sensitive to pK_{a4} , although the reaction rates of K289M are slower than what is observed for WT. For the dihalogenated analogues, β, γ -CF₂ dCTP binds to WT and K289M with similar affinities but K289M has a 10-fold lower k_{pol} (Tables 1 and 2). The β, γ -CFCl dCTP binds more tightly to both the WT and K289M pol β than the parent dCTP does, and the k_{pol} is 10-fold lower for K289M (Tables 1 and 2). The β, γ -CCl₂ dCTP analogue is intriguing because it exhibits similar k_{pol} values and weak binding for both WT and K289M; the Brønsted correlation lines for WT and K289M converge at this point (Figure 4). The sensitivity to leaving group pK_{a4} of the incorrect dihalogenated analogues results in steep slopes of -1.7 for WT and -1.0 for K289M, consistent with a RDS dependent on chemistry sensitive to leaving group charge, though with different degrees of charge buildup at the TS.

The reaction rates of WT and K289M display a similar dependence on the pK_{a4} for the other analogues. The slope from the Brønsted correlation is -0.6 for both the WT and K289M. The polymerization rates catalyzed by K289M are consistently 10-fold slower than those of WT, resulting in similar LFER slopes. Additionally, the binding affinity of WT for the analogues is similar to that of K289M, with the exception of β, γ -CHBr, which binds with better affinity to WT. The dependency of the reaction rates on the pK_{a4} for K289M suggests that the charge buildup at the TS for this variant is similar to that of WT pol β for incorrect incorporation.

K289M Becomes More Accurate in the Presence of the Analogues

As this mutant is a fidelity mutant, we compared the fidelity of the WT and K289M enzymes for the analogue substrates and also for the analogues versus the parent dNTP (Table 3). In comparison to those of the parent compounds, the fidelity of both WT and K289M is increased with each of the analogues (Table 3, column 4). The increase in fidelity observed between the analogues and the parent compound is greatest for K289M (Table 3, column 4, $F_{analogue/parent}$). For WT, fidelity is increased ~ 1.5 – 2.5 -fold with the analogues compared to that with the parent compound, the exception being CCl₂ for which the increase is ~ 37 -fold. However, the fidelity of K289M is increased 12–22-fold versus that of the parent compound (Table 3, column 4, $F_{analogue/parent}$) in most cases, the exceptions being CFCl and CHF, for which the observed increase in fidelity is only ~ 3 – 6 -fold. For the majority of the analogues, the mechanistic basis for the increased fidelity of K289M is discrimination predominantly at the level of substrate binding (Table 4). When compared to K289M, WT exhibits an increased level of discrimination for the analogues themselves at the level of k_{pol} . However, K289M exhibits an increased level of discrimination over what is observed for WT (analogue/parent) at the level of k_{pol} (Table 5). Therefore, in most cases, the fidelity of K289M is greatly increased when the analogues, rather than the parent compound, are substrates for polymerization.

Discussion

In this study, we show that the rate of nucleotidyl transfer of the K289M colon cancer-associated mutator variant of pol β is significantly less dependent upon the pK_{a4} of the leaving group than that of WT pol β for incorporation of the correct dNTP analogue. In

addition, K289M exhibits a stronger dependence on the pK_{a4} of the leaving group for incorporation of the incorrect dCTP analogues versus the correct dGTP analogues. However, K289M is less dependent on the pK_{a4} of the leaving groups of the incorrect dihalogenated dCTP analogues than WT pol β is. In combination, our results suggest that one of the relative rate constants differs for a step along the reaction coordinate of K289M compared to that of WT pol β in a manner different than for the incorporation of the correct dGTP analogue at $pK_{a4} < 10.5$, which like dCTP analogue incorporation by K289M is more similar to that of WT pol β .

We also show that the fidelity of K289M increases in the presence of several dNTP analogues, and that the discrimination at the level of dNTP binding is also increased (Table 4). This suggests that alteration of Lys289 to Met results in an enzyme with a significantly altered dNTP binding pocket that facilitates selection of correct dNTPs carrying specific modifications of β, γ -CXY bridging groups.

A Change at Lys289 Leads to a Change in One or More of the Relative Rates of the Mechanism for Correct Incorporation

Results from our work demonstrate a negative dependence of the TS for correct incorporation by WT on the leaving group pK_{a4} of the dNTP analogue substrate, as indicated by the LFER slopes (β is -0.6 for dihalogenated and -0.4 for other substituted analogues) (Figure 3), consistent with a TS in which leaving group departure is implicated, as previously proposed.^{13,42,50,55,59}

Our work focusing on the K289M cancer-associated variant shows that the mutant enzyme has a significantly decreased dependence on the leaving group pK_{a4} for the correct dGTP analogues, as shown by the LFER slopes (-0.25 for dihalogenated and -0.04 for other substituted analogues) (Figure 3). This suggests that K289M catalyzes polymerization of correct dNTPs via the formation of a TS that is less dependent on charge differences in the leaving group. Although Lys289 is located at a distal location from the active site, removing the positively charged lysine and replacing it with a neutral methionine alters the kinetic phenotype, i.e., reaction pathway, of the enzyme predominantly for correct incorporation. More specifically, Lys289 is located in the fingers subdomain on helix N (residues 275–289). This particular helix has been shown by time-resolved X-ray crystallography to move closer to the minor groove of the DNA upon binding of the correct dNTP. Our previous FRET studies using WT pol β also provide evidence that one or more conformational changes occur during correct dNTP incorporation of dGTP opposite template C in the primer-template substrate T(–8)D:3'OH. This is the DNA sequence utilized in the majority of crystal structures of pol β , with the exception of a Dabcyl label at position –8 in the template and not the DNA primer-template used in this study (Scheme 1).³²

Knowing that the alteration of Lys289 to Met does not significantly change K_d (Table 2) and that the alteration of Lys to Met results in product formation independent of charge differences in the leaving group, we speculate that there is a change in the equilibrium constant for one of these conformational changes that precedes chemistry.

Lys289 is located at the distal end of the helix, away from the active site (Figure 5). Molecular dynamics simulations show that the ϵ -amino group of Lys289 forms a salt bridge with Glu288 in the binary structure and with Gln324 in the ternary complex, which makes it important for the formation of the ternary complex and the alignment of the active site for activity⁶⁰ (Figure 5). The crystal structure also shows that Lys289 moves 12 Å closer to the terminal phosphate of the downstream strand of the double-stranded DNA. This long-distance DNA interaction with the positively charged Lys289 may be important for the closing of the fingers subdomain to prepare the active site for chemistry. The loss of the positively charged Lys289 may destabilize the closing of the fingers subdomain upon dNTP binding, making the reaction independent of the β,γ -bridging substitution for $pK_a < 10.5$, and reducing activity.

WT and K289M Exhibit Similar Relative Rates along the Reaction Coordinate for Misincorporation

Both WT and K289M enzymes have a large negative LFER slope for misincorporation ($WT_{\text{other}}, -0.6$; $WT_{\text{dihalogenated}}, -1.7$; $K289M_{\text{other}}, -0.6$; $K289M_{\text{dihalogenated}}, -1.0$) (Figure 4). The slope suggests that chemistry is rate-limiting for both WT and K289M and that the TS has a highly charged characteristic that is very sensitive to leaving group pK_a . The RDS for WT and K289M during the incorrect incorporation of C opposite templating C has not been previously investigated, and we provide evidence consistent with it being a chemical step. Previous work from our lab and others indicates that one or more conformational changes preceding chemistry may be very slow for incorporation of the incorrect dNTP and may approach reaction rates measured in this study.^{32,44,61,62}

Our previous FRET studies, in addition to recent NMR work, suggest that the closing of the fingers subdomain is most stable during correct incorporation and is destabilized during misincorporation.^{32,33} Closing of the fingers subdomain is proposed to align the active site for the inline attack, which makes it important for the formation of the TS. The rate of reverse closing is in fact significantly slower than the rate of chemistry during correct nucleotide incorporation, which commits the enzyme to chemistry.³² This is consistent with previous reports about HIV RT and T7 polymerase.^{34,35,38} Our recent work suggests that misincorporation may occur in the absence of one or more stable conformational changes.³² Thus, if the K289M mutation destabilizes the formation of the closed ternary complex, the mutation will not be limiting for misincorporation and will limit only correct dNTP incorporation.

The Analogues Improve the Fidelity of K289M

We show that most of the analogues rescue the low-fidelity phenotype of K289M (Table 3). Although the K289M cancer variant exhibits less discrimination than WT does at the k_{pol} level (Table 5), the analogues improve discrimination at the level of ground state binding for K289M (Table 4). This results predominantly from the improved affinity of K289M versus that of WT for the correct analogues as WT and K289M exhibit similar affinities for the incorrect analogues. Although the activity is low, the higher binding affinity for the correct analogues commits the enzyme to catalyze the correct incorporation.

One of the hallmarks of fingers closing is the repositioning of Arg183 to form a hydrogen bond with $P\beta$ of the incoming correct nucleotide.^{63,64} If the fingers subdomain in K289M does not close in a stable manner, the Arg183 residue is proposed to be distant from the bound dNTP. Therefore, if the bridging groups potentially cause steric clashes with Arg183, this will not occur with K289M if it is unable to form a stable closed structure. This could explain why there is increased discrimination for the correct analogues at the level of dNTP binding in the presence of Met289. Alternatively, the dNTP binding pocket of K289M may be altered in some other manner that promotes tighter binding of the correct dNTP analogues.

Mutator variants have the ability to drive carcinogenesis, and we have previously shown that expression of K289M in immortal but nontransformed cells induces cellular transformation.¹⁷ Increased levels of mutagenesis induced by a mutator polymerase also have the ability to promote resistance to chemotherapeutic drugs that affect DNA. Specific dNTP analogues targeting enzymes that drive a mutator phenotype in tumors may be useful in limiting mutagenesis that leads to drug resistance, a hypothesis that will be tested in future experiments.

Conclusions

In summary, we show that a single mutation in pol β can lead to changes in the relative rate constants for catalysis of correct incorporation of dGTP. This K289M mutation may slow prechemical conformational changes upon dNTP binding that make the rate of product formation less dependent on variation in the basicity of the leaving group. For incorrect incorporation, the K289M and WT enzymes exhibit similar dNTP binding affinities, and their rate constants show a similar dependence on the pK_{a4} of the leaving group in the dNTP analogues. This unexpected result demonstrates that despite its distal position relative to the active site in the ground state conformation of the protein, Lys289Met modulates the relative rate constants for correct and incorrect incorporation in different ways, suggesting that it (and possibly other pol β variants) is a plausible target for therapeutic inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Vinod Batra and Samuel Wilson for Figure 5.

Funding: Supported by National Cancer Institute Research Program U19 CA177547.

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Abbreviations

APC	adenomatous polyposis coli
BER	base excision repair
dRP	deoxyribose 5'-phosphate
dNTP	deoxynucleoside 5'-triphosphate
K_d	dissociation constant for dissociation of dNTP from polymerase β
FRET	fluorescence resonance energy transfer
k_{pol}	rate constant for introduction of a nucleotide into gapped DNA
LFER	linear free energy relationship
PP_i	inorganic pyrophosphate
RDS	rate-determining step
TEAB	triethylammonium bicarbonate
TS	transition state
WT	wild type

Figure 1.
 β,γ -dGTP and β,γ -dCTP analogues,

Figure 2.

Single-turnover kinetics for the incorporation of the correct β,γ -CH₂ dGTP. Single-turnover kinetics of product formation by (A and B) WT and (C and D) K289M in the presence of varying concentrations of dGTP β,γ -CH₂ opposite template C (blue, 0.25 μ M; red, 0.5 μ M; green, 1 μ M; purple, 2.5 μ M; orange, 5 μ M; black, 10 μ M; brown, 25 μ M). The single-gap DNA substrate used for these reactions is shown at the top. The templating dC is underlined and shown in bold. Pol β and the CL-CP-CG DNA template with a single-nucleotide gap (750 and 50 nM, respectively) were premixed, and reactions were initiated upon mixing with dGTP on the Kin-Tek rapid quench-flow apparatus for various times at 37 °C. Reactions were quenched with EDTA, and the product was separated by PAGE. Bands were quantified, and product formed was plotted as a function of time. Points were fitted to a single-exponential equation to obtain rates (k_{obs}) at each dGTP concentration. Rates were plotted as a function of dGTP β,γ -CH₂ concentration and fitted with a hyperbolic equation to obtain k_{pol} and K_{d} for (B) WT and (D) K289M. The leaving group $\text{p}K_{\text{a4}}$ of dGTP β,γ -CH₂ is 10.5, which is much higher than that of the parent dGTP. K_{d} values of WT ($7.1 \pm 1.2 \mu\text{M}$) and K289M ($1.7 \pm 0.1 \mu\text{M}$) are unaffected by the high $\text{p}K_{\text{a4}}$. The k_{pol} of WT is much slower than that of the parent ($10.1 \pm 0.7 \text{ s}^{-1}$), but that of K289M was unaffected ($0.7 \pm 0.0 \text{ s}^{-1}$).

Figure 3.

Brønsted correlation plots for correct nucleotide insertion. Brønsted correlation plot of $\log(k_{\text{pol}})$ vs leaving group pK_{a4} for the correct incorporation of dGTP analogues opposite template C by WT (blue) and K289M (red) Pol β . Points were fitted with the equation of a line to generate the slopes. (A) Additional methylated and monohalogenated analogues show similar patterns at $pK_{a4} < 10.5$ (WT, -0.4; K289M, -0.04). A break in the line occurs at $pK_{a4} > 10.5$, representing a change in the RDS for both WT and K289M (WT, -1.3; K289M, -0.78). (B) Incorporation of dihalogenated dGTP analogues by K289M is only weakly dependent on pK_{a4} , as shown by the shallow slope of -0.25 compared to that of WT (-0.63).

Figure 4.

Brønsted correlation plots for incorrect nucleotide insertion. Brønsted correlation plots for the incorporation of the incorrect dCTP analogues opposite template C for WT (blue) and K289M (red) pol β . The relationship for the K289M variant mimics that of the WT. The relationship is linear, and both have increased sensitivity to pK_{a4} compared to that for correct insertion (WT_{other}, -0.6; WT_{dihalogenated}, -1.7; K289M_{other}, -0.6; K289M_{dihalogenated}, -1.0).

Figure 5.

Active site assembly of WT human DNA pol β and the location of Lys289. α -Helix N closes over the incoming nucleotide, in this case (R)- β , γ -fluoromethylene-dGTP. The ternary complex (gold) positions the nascent base pair (templating base and the incoming nucleotide), so that it is sandwiched between the primer terminus and α -helix N side chains. In the closed (ternary) conformation, Arg283 (R283) located in the middle of α -helix N interacts (dashed line) with the sugar of the Tn-1 templating strand nucleotide. The methylene side chains of Asp276 (D276) and Lys280 (K280) stack with the bases of the incoming and templating nucleotides, respectively. Asp190 (D190), Asp192 (D192), and Asp256 (D256) coordinate the metal ions in the active site, and several other interactions are also shown. Lys289 (K289) forms a salt bridge with Gln324 (Q324) in the ternary complex, and this stabilizes α -helix N in the closed conformation. The K289M variant enzyme could have an alteration in the position of α -helix N in the closed ternary complex. Protein Data Bank codes for the binary and ternary complex crystal structures are 1BPX and 2PXI, respectively.

Scheme 1. Biochemical Pathway for Nucleotide Incorporation by WT Pol β in the Presence of the Crystal Structure Primer–Template Substrate T(–8)D:3'OH^a

^aThe binary complex, β -DNA_n, binds dNTP with a specific $K_{d(dNTP)}$ (step 2). Binding of dNTP leads to conformational changes (steps 3 and 3.1), which align the active site for nucleophilic attack. The rate of DNA polymerization is designated as step 3.2. Finally, product DNA (DNA_{n+1}) is released in step 4. This reaction pathway was adapted from ref 32.

Table 1

Summary of WT Kinetic Data from Single-Turnover Activity^a

M-N ^b	X- ^b	pK _{as} ^c	k _{pol} (s ⁻¹)	K _d (μM)	efficiency (μM ⁻¹ s ⁻¹) ^d
C-G	CF ₂	7.8	43.6 ± 3.0	7.8 ± 1.4	5.6 ± 1.0
C-G	CFCI	8.4	22.8 ± 1.2	3.4 ± 0.5	6.7 ± 1.0
C-G	CCl ₂	8.8	18.4 ± 1.2	51.0 ± 8.0	0.4 ± 0.1
C-G	O	8.9	27.6 ± 1.2	2.3 ± 0.3	12.0 ± 1.9
C-G	CHF	9.0	36.4 ± 2.0	7.5 ± 0.7	4.9 ± 0.5
C-G	CBF ₂	9.3	3.7 ± 0.2	106 ± 12	0.03 ± 0.00
C-G	CHCl	9.5	20.3 ± 1.8	7.8 ± 1.6	2.6 ± 0.5
C-G	CH ₂	10.5	10.1 ± 0.7	7.1 ± 1.2	1.4 ± 0.3
C-G	CHCH ₃	11.6	0.2 ± 0.0	2.0 ± 0.4	0.08 ± 0.00
C-G	C(CH ₃) ₂	12.3	0.03 ± 0.00	195 ± 50	(1.3 ± 0.2) × 10 ⁻⁴
C-C	CF ₂	7.8	0.10 ± 0.01	845 ± 158	(1.2 ± 0.2) × 10 ⁻⁴
C-C	CFCI	8.4	0.02 ± 0.00	103 ± 24	(1.5 ± 0.3) × 10 ⁻⁴
C-C	CCl ₂	8.8	0.001 ± 0.000	1678 ± 499	(5.9 ± 2.5) × 10 ⁻⁷
C-C	O	8.9	0.22 ± 0.00	340 ± 68	(6.5 ± 1.3) × 10 ⁻⁴
C-C	CHF	9.0	0.09 ± 0.00	690 ± 46	(1.4 ± 0.1) × 10 ⁻⁴
C-C	CHCl	9.5	0.07 ± 0.01	705 ± 125	(9.5 ± 1.8) × 10 ⁻⁵
C-C	CHBr	9.9	0.04 ± 0.00	82 ± 30	(5.1 ± 0.2) × 10 ⁻⁴
C-C	CH ₂	10.5	0.02 ± 0.00	445 ± 74	(3.6 ± 0.6) × 10 ⁻⁵

^a Values are reported as means ± the standard deviation of three or more repeats.^b M-N is the template-incoming dNTP, and -X- is the β-γ-bridging group.^c pK_{as} values from ref 50.^d Efficiency is k_{pol}/K_d.

Table 2
Summary of K289M Kinetic Data from Single-Turnover Activity^a

M-N ^b	X- ^b	pK _{as} ^c	k _{pol} (s ⁻¹)	K _d (μM)	efficiency (μM ⁻¹ s ⁻¹) ^d
C-G	CF ₂	7.8	0.8 ± 0.0	1.0 ± 0.2	0.9 ± 0.1
C-G	CFCI	8.4	0.8 ± 0.1	1.3 ± 0.3	0.6 ± 0.1
C-G	CCl ₂	8.8	0.7 ± 0.1	25 ± 7	0.03 ± 0.01
C-G	O	8.9	0.9 ± 0.1	1.3 ± 0.3	0.7 ± 0.2
C-G	CHF	9.0	0.9 ± 0.0	1.6 ± 0.2	0.5 ± 0.0
C-G	CBF ₂	9.3	0.3 ± 0.0	73 ± 10	(4.5 ± 0.6) × 10 ⁻³
C-G	CHCI	9.5	0.8 ± 0.1	2.0 ± 0.4	0.4 ± 0.1
C-G	CH ₂	10.5	0.7 ± 0.0	1.7 ± 0.1	0.4 ± 0.0
C-G	CHCH ₃	11.6	0.13 ± 0.01	7.0 ± 1.0	0.02 ± 0.00
C-G	C(CH ₃) ₂	12.3	0.02 ± 0.00	446 ± 73	(3.4 ± 0.6) × 10 ⁻⁵
C-C	CF ₂	7.8	0.01 ± 0.00	882 ± 253	(1.1 ± 0.2) × 10 ⁻⁵
C-C	CFCI	8.4	0.003 ± 0.000	65 ± 13	(5.1 ± 1.0) × 10 ⁻⁵
C-C	CCl ₂	8.8	0.001 ± 0.000	1521 ± 1176	(6.6 ± 5.7) × 10 ⁻⁷
C-C	O	8.9	0.05 ± 0.00	267 ± 88	1.9 × 10 ⁻⁴ ± 0.7 × 10 ⁻⁷
C-C	CHF	9.0	0.01 ± 0.00	572 ± 65	(2.3 ± 0.3) × 10 ⁻⁵
C-C	CHCI	9.5	0.007 ± 0.001	1062 ± 208	(6.1 ± 1.3) × 10 ⁻⁶
C-C	CHBr	9.9	0.003 ± 0.000	638 ± 59	(5.9 ± 0.1) × 10 ⁻⁵
C-C	CH ₂	10.5	0.004 ± 0.000	615 ± 128	(6.3 ± 1.4) × 10 ⁻⁶

^a Values are reported as means ± the standard deviation of three or more repeats.

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

^c pK_{as} values from ref 50.

^d Efficiency is k_{pol}/K_d.

Table 3

Fidelity of WT and K289M

enzyme	-X-	p <i>K</i> _{s4}	<i>F</i> analogue ^d	<i>F</i> analogue/parent ^b	K289M/WT analogue ^c	K289M/WT (analogue/parent) ^d
WT	CF ₂	7.8	46668	2.5		
	CFCI	8.4	44668	2.4		
	CCl ₂	8.8	677967	36.7		
	O	8.9	18463	1.0		
	CHF	9.0	35001	1.9		
K289M	CHCI	9.5	27369	1.5		
	CH ₂	10.5	38890	2.1		
	CF ₂	7.8	81819	22.2	1.8	8.8
	CFCI	8.4	11766	3.2	0.3	1.3
	CCl ₂	8.8	45456	12.3	0.1	0.3
	O	8.9	3685	1.0	0.2	1.0
	CHF	9.0	21740	5.9	0.6	3.1
	CHCI	9.5	65575	17.8	2.4	12.0
	CH ₂	10.5	63493	17.2	1.6	8.2

^a Fidelity, *F*, is calculated from [efficiency(correct) + efficiency(correct) / efficiency(incorrect)].

^b $F_{\text{analogue}}/F_{\text{parent}}$.

^c $[F_{\text{K289M}}(\text{analogue})] / [F_{\text{WT}}(\text{analogue})]$

^d $[F_{\text{K289M}}(\text{analogue/parent})] / [F_{\text{WT}}(\text{analogue/parent})]$.

Table 4

Discrimination at K_d by WT and K289M

enzyme	-X-	p <i>K_a</i> ^a	D <i>K_d</i> (i/c) analogue ^a	D <i>K_d</i> analogue/parent ^b	D <i>K_d</i> analogue K289M/WT ^c
WT	CF ₂	7.8	108.3	0.7	
	CFCI	8.4	30.3	0.2	
	CCl ₂	8.8	32.9	0.2	
	O	8.9	147.8	1.0	
	CHF	9.0	92.0	0.6	
K289M	CHCl	9.5	90.4	0.6	
	CH ₂	10.5	62.7	0.4	
	CF ₂	7.8	882.0	4.3	8.1
	CFCI	8.4	50.0	0.2	1.7
	CCl ₂	8.8	60.8	0.3	1.8
	O	8.9	205.4	1.0	1.4
	CHF	9.0	357.5	1.7	3.9
	CHCl	9.5	531.0	2.6	5.9
	CH ₂	10.5	361.8	1.8	5.8

^aDiscrimination, D, at K_d is calculated from $K_d(\text{incorrect})/K_d(\text{correct})$.^bD K_d analogue/D K_d parent.^cD K_d K289M analogue/D K_d WT analogue.

Table 5

Discrimination at k_{pol} by WT and K289M

enzyme	X-	$pK_{a,4}$	D $k_{\text{pol}}(c/i)$ analogue ^d	D k_{pol} analogue/parent ^b	D k_{pol} analogue WT/K289M ^c
WT	CF ₂	7.8	436	3.5	5.5
	CFCI	8.4	1140	9.1	4.3
	CCl ₂	8.8	18400	146.7	26.3
	O	8.9	125	1.0	7.0
	CHF	9.0	404	3.2	4.5
K289M	CHCl	9.5	290	2.3	2.5
	CH ₂	10.5	505	4.0	2.9
	CF ₂	7.8	80	4.4	
	CFCI	8.4	267	14.8	
	CCl ₂	8.8	700	38.9	
	O	8.9	18	1.0	
	CHF	9.0	90	5.0	
	CHCl	9.5	114	6.3	
	CH ₂	10.5	175	9.7	

^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} K289M analogue.