Substitution of a residue contacting the triphosphate moiety of the incoming nucleotide increases the fidelity of yeast DNA polymerase ζ

Craig A. Howell, Christine M. Kondratick and M. Todd Washington*

Department of Biochemistry, University of Iowa College of Medicine, Iowa City, IA 52242-1109, USA

ABSTRACT

DNA polymerase ζ (pol ζ), which is required for DNA damage-induced mutagenesis, functions in the error-prone replication of a wide range of DNA lesions. During this process, pol ζ extends from nucleotides incorporated opposite template lesions by other polymerases. Unlike classical polymerases, pol ζ efficiently extends from primer-terminal base pairs containing mismatches or lesions, and it synthesizes DNA with moderate fidelity. Here we describe genetic and biochemical studies of three yeast pol ζ mutant proteins containing substitutions of highly conserved amino acid residues that contact the triphosphate moiety of the incoming nucleotide. The R1057A and K1086A proteins do not complement the rev3Δ mutation, and these proteins have significantly reduced polymerase activity relative to the wild-type protein. In contrast, the K1061A protein partially complements the rev3Δ mutation and has nearly normal polymerase activity. Interestingly, the K1061A protein has increased fidelity relative to wild-type pol ζ and is somewhat less efficient at extending from mismatched primer-terminal base pairs. These findings have important implications both for the evolutionary divergence of pol ζ from classical polymerases and for the mechanism by which this enzyme accommodates distortions in the DNA caused by mismatches and lesions.

INTRODUCTION

DNA replication by classical DNA polymerases—those involved in the replication of non-damaged DNA—is blocked by many types of distorting DNA lesions. One means of overcoming replication blocks at sites of DNA damage is translesion DNA synthesis (TLS) by specialized DNA polymerases. In yeast, the *RAD30* gene, which encodes specialized DNA polymerase η (pol η), functions in error-free TLS of cyclobutane pyrimidine dimers and 8-oxoguanines (1,2). Inactivation of pol η in yeast increases the frequency of UV-induced mutagenesis (3–5) and in humans, causes the cancer-prone disorder the variant form of xeroderma pigmentosum (6,7). The yeast *REV3* gene, which encodes the catalytic subunit of DNA polymerase ζ (pol ζ), functions in error-prone TLS of a wide range of DNA lesions (8–12). Inactivation of *REV3* in yeast results in a dramatic decrease in the rate of DNA damage-induced mutagenesis (13–15). In addition, human cells expressing anti-sense RNA to this gene have significantly reduced UV-induced mutation rates (16).

Pol ζ is a heterodimer comprised of a 173 kDa catalytic subunit encoded by the *REV3* gene and a 29 kDa accessory subunit encoded by the *REV7* gene (8,9). While classical polymerases synthesize DNA with high fidelity, pol ζ synthesizes DNA with moderate fidelity. Its frequency of misincorporation (f_{inc}) ranges from 10^{-3} to 10^{-5} (17), which is an order of magnitude lower fidelity than classical mammalian pol α ($f_{inc} \sim 10^{-4}$–10^{-6}) (18) and an order of magnitude or so higher fidelity than specialized pol η ($f_{inc} \sim 10^{-2}$–10^{-3}) (19–21). However, the most striking difference between pol ζ and classical DNA polymerases is its ability to extend from primer-terminal base pairs containing mismatches. The relative efficiencies of extending from mismatched versus matched primer termini (f_{ext}^0) for pol ζ ranges from 10^{-1} to 10^{-2} (17), which is several orders of magnitude greater than for pol α ($f_{ext}^0 \sim 10^{-3}$–10^{-6}) (18), and an order of magnitude greater than pol η (f_{ext}^0 10^{-2}–10^{-3}) (22). Moreover, this promiscuous misextension ability allows pol ζ to efficiently extend from primer-terminal base pairs containing template DNA lesions, including cyclobutane pyrimidine dimers, 6–4 photoproducts, and abasic sites (17). These abilities of pol ζ are essential to its role in TLS, because pol ζ is believed to function by extending from nucleotides incorporated opposite template DNA lesions by other polymerases (23,24).

*To whom correspondence should be addressed. Tel: +1 319 335 7518; Fax: +1 319 335 9570; Email: todd-washington@uiowa.edu

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

Like several classical eukaryotic polymerases—such as pol α, pol δ and pol ε—pol ζ is a member of the class B family of DNA polymerases (25). High-resolution structures currently exist for two class B polymerases: the bacteriophage RB69 DNA polymerase (26,27) and the bacteriophage φ29 DNA polymerase (28). Like polymerases from other families, these enzymes contain palm, thumb and fingers subdomains. The palm sub-domain is a five-stranded β-sheet containing conserved acidic residues that coordinate the two metal ions necessary for catalysis. The thumb subdomain is pre-dominantly α-helical and makes extensive contacts with the duplex region of the DNA. The fingers subdomain is entirely α-helical, and it interacts intimately with the incoming dNTP.

Comparisons of X-ray crystal structures of various classical DNA polymerases in the presence and absence of DNA and dNTP have shown that the fingers subdomain plays a critical role in maintaining polymerase fidelity (29–31). When the enzyme is bound to DNA, but not to an incoming dNTP, the fingers subdomain adopts an 'open' conformational state. When a correct dNTP binds, the fingers subdomain adopts a 'closed' conforma-tional state. The α-phosphate of the incoming dNTP, the 3′OH of the primer strand, and the two metal ions are properly positioned for catalysis only in the 'closed' conformation. The incorporation of incorrect dNTPs is inefficient because the binding pocket for the nascent base pair (i.e. the base pair containing the incoming dNTP) in the 'closed' state is too tight to accommodate mismatches (29–31). Although no high-resolution struc-tures have yet been determined for a DNA polymerase bound to an incorrect dNTP, the structures of complexes approximating this state have been determined (32,33). They show that in the presence of a geometrically distorted nascent base pair, the fingers subdomain adopts a 'partially open' conformation that is not conducive to catalysis.

To better understand the contribution of individual amino acid residues to the remarkable enzymatic proper-ties of pol ζ, we carried out genetic and biochemical studies of pol ζ mutant proteins. We have focused on three amino acid residues in the fingers subdomain that interact with the triphosphate moiety of the incoming dNTP and are completely conserved among the class B family polymerases. Two of these mutant proteins, R1057A and K1086A, have significantly reduced DNA polymerase activity compared to wild-type pol ζ. In contrast, the DNA polymerase activity of one mutant protein, K1061A, was not significantly reduced relative to wild-type. Steady-state kinetic studies of this mutant protein revealed that it has an increased fidelity relative to wild-type pol ζ and a somewhat decreased ability to extend from mismatched primer-terminal base pairs. Thus, Lys-1061 plays a critical role in accommodating distortions in the DNA at both the nascent base pair and the primer-terminal base pair. These findings have important implications both for the evolutionary divergence of pol ζ from classical poly-merases and for the mechanism by which this enzyme accommodates distortions in the DNA caused by mis-matches and lesions.

MATERIALS AND METHODS

Site-directed mutagenesis and plasmid constructions

To generate the pol ζ mutant proteins, we used the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) to introduce base substitutions into pKW001 (pUC19 containing wild-type *REV3*) to generate pKW007 (K1061A), pKW008 (R1057A) and pKW009 (K1086A). We next used PCR to amplify the 500 bp of DNA immediately 5′ of the *REV3* gene in the yeast genome, and this region of genomic DNA containing the native *REV3* promoter was cloned upstream of the wild-type and mutant genes in these vectors. The wild-type and mutant genes with 500 bp of upstream genomic DNA were then subcloned into the low-copy-number *CEN/ARS LEU2* vector YCplac111 (34) to generate pKW035 (R1057A), pKW036 (K1061A), pKW037 (K1086A) and pKW039 (wild-type) for genetic complementation studies.

To over-express the wild-type and mutant proteins, we cloned these genes into pKW144, a 2 μm shuttle vector with a *leu2-d* selectable marker, in frame with a protease-cleavable glutathione-s-transferase (GST) gene under control of the hybrid *GAL PGK* promoter to generate pKW017 (R1057A), pKW018 (K1061A), pKW019 (K1086A) and pKW040 (wild-type).

Genetic complementation studies

We tested the wild-type and mutant pol ζ proteins for biological function *in vivo* by examining their ability to complement the UV sensitivity of the *rev3Δ* strain (12). Strains harboring plasmids pKW035, pKW036, pKW037 and pKW039 (*CEN/ARS LEU2* YCplac111-derived vectors containing the wild-type and mutant genes) were grown to mid-exponential phase in liquid synthetic complete medium lacking leucine. Cells were washed and diluted in water and plated on synthetic complete medium lacking leucine. The plates were then exposed to UV radiation and incubated in the dark at 30°C. Colonies were counted after 3–5 days.

We also tested the *in vivo* function of the wild-type and mutant pol ζ proteins by examining their ability to support UV-induced mutagenesis of the *rev3Δ* strain. Cells were treated as before, except that they were plated on synthetic complete medium lacking leucine to determine viability and on synthetic complete medium lacking arginine, but containing canavanine to determine the frequency of $CAN1^S$ to $can1^r$ forward mutations.

Purification of pol ζ

Yeast strains BJ5464 harboring plasmids pKW017, pKW018, pKW019 or pKW040 (2 μm *leu2-d* vectors encoding GST-pol ζ wild-type and mutant proteins under the *GAL PGK* promoter) and pKW141, a 2 μm *URA3* vector containing the wild-type REV7 gene

also under the *GAL PGK* promoter, were used for over-expressing wild-type and mutant pol ζ as described previously (17). Both wild-type and mutant proteins were purified as GST-fusion proteins in similar yields using protocols previously published for yeast pol η (35). During purification, the GST portion of the fusion protein was removed by treatment with PreScission protease (Amersham Pharmacia), and protein samples were stored in aliquots at −80°C.

Preparation of DNA substrates

For the DNA polymerase activity assays, synthetic 45-mer oligodeoxynucleotide with the sequence 5′-GGA CGG CAT TGG ATC GAC CT<u>N</u> GAG TTG GTT GGA CGG CTG CGA GGC, where <u>N</u> can be G, A, T or C, were used as template strands. For the fidelity measurements, a synthetic 24-mer oligodeoxynucleotide with the sequence: 5′-GCC TCG CAG CCG TCC AAC CAA CTC, was used as a primer strand. For the mismatch extension measurements, synthetic 25-mer oligodeoxynucleotides with the sequence: 5′-GCC TCG CAG CCG TCC AAC CAA CTC <u>N</u>, where <u>N</u> can be G, A, T or C, were used as primer strands. Primer strands were 5′-^{32}P-end-labeled using T4 polynucleotide kinase (New England Biolabs) and (^{32}P-γ)ATP (PerkinElmer). Template strands and labeled primer strands (1 μM each) were annealed in 25 mM Tris–Cl, pH 7.5, 100 mM NaCl by heating to 90°C for 2 min and slow cooling to 22°C over several hours. Labeled and annealed DNA substrates were stored at 4°C for up to 1 week.

Steady-state kinetic studies

Wild-type or mutant pol ζ (1 nM) was incubated with 20 nM of the DNA substrate and various concentrations of one of the four dNTPs (0–2 mM) in the presence of 25 mM Tris–Cl, pH 7.5, 5 mM MgCl$_2$, 5 mM dithiothreitol, 100 μg/ml bovine serum albumin and 10% glycerol at 22°C. Reactions were quenched after various times up to 90 min by the addition of 10 volumes of formamide loading buffer (80% deionized formamide; 10 mM EDTA, pH 8.0; 1 mg/ml xylene cyanol; 1 mg/ml bromophenol blue). Extended primer strands (the product) and unextended primer strands (the substrate) were separated on a 15% polyacrylamide sequencing gel containing 8 M urea. The intensities of the labeled gel bands were determined using the InstantImager (Packard).

The rate of product formation, determined by dividing the amount of product formed by the reaction time, was graphed as a function of dNTP concentration, and the k_{cat} and K_m values were determined from the best fit of the data to the Michaelis–Menten equation using non-linear regression (SigmaPlot 8.0). The frequency of misincorporation (f_{inc}) was determined using the following formula (36,37):

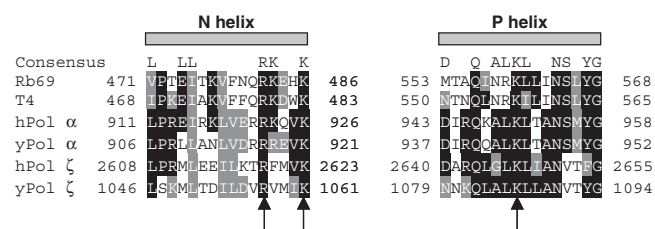$$f_{inc} = \frac{(k_{cat}/K_m)_{incorrect}}{(k_{cat}/K_m)_{correct}}$$

Figure 1. Alignment of amino acid residues from two conserved regions of several class B family DNA polymerases. Amino-acid sequences of the bacteriophage RB69 DNA polymerase, the bacteriophage T4 DNA polymerase, human pol α, yeast pol α, human pol ζ and yeast pol ζ are shown. Black boxes represent identical amino acid residues, and gray boxes represent similar amino-acid residues. The secondary structural elements and the consensus sequences are from reference (26). Arrows indicate the amino acid residues corresponding to Arg-1057, Lys-1061 and Lys-1086 in yeast pol ζ.

where 'correct' refers to the values for correct dNTP incorporation and 'incorrect' refers to the values for incorrect dNTP incorporation. The relative frequency of mismatch extension (f_{ext}^0) was determined using the following formula (36,37):

$$f_{ext}^0 = \frac{(k_{cat}/K_m)_{mismatched}}{(k_{cat}/K_m)_{matched}}$$

where 'matched' refers to the values for correct dNTP incorporation following a matched primer-terminal base pair and 'mismatched' refers to incorporation following a mismatched primer-terminus.

RESULTS

To examine the contribution of individual amino-acid residues to the enzymatic properties of pol ζ, we characterized several pol ζ mutant proteins generated by site-directed mutagenesis. We initially decided to focus on residues in the fingers subdomain that interact with the triphosphate moiety of the incoming dNTP. To identify such residues, we carried out an amino-acid sequence alignment with various members of the class B family of DNA polymerases including the bacteriophage RB69 DNA polymerase for which a high-resolution structure has been determined (26,27). Figure 1 shows the alignment of two conserved motifs, corresponding to portions of helices N and P in the fingers subdomain of the RB69 polymerase. We selected Arg-1057, Lys-1061 and Lys-1086—which correspond to Arg-482, Lys-486 and Lys-560 in RB69 polymerase, respectively—for site-directed mutagenesis. These residues were shown to interact with the triphosphate moiety of the incoming dNTP in the X-ray crystal structure of the RB69 polymerase bound to both DNA and dNTP (27), and these residues are conserved completely among the class B family of DNA polymerases.

Genetic analyses of the pol ζ mutant proteins

We carried out genetic analyses to assay the function of the pol ζ mutant proteins *in vivo*. We first assayed them for the ability to complement the sensitivity of the *rev3Δ* yeast
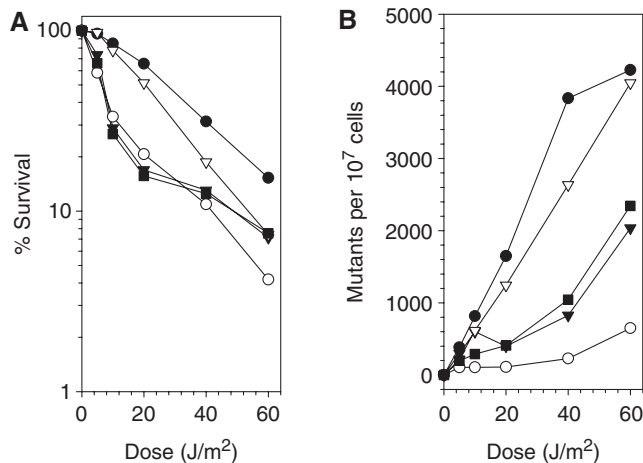
Figure 2. Genetic complementation analysis of the pol ζ mutant proteins. (**A**) The UV sensitivity of yeast strains lacking pol ζ (*rev3Δ*) harboring plasmids producing either no pol ζ (open circle), wild-type pol ζ (filled circle), the R1057A mutant protein (inverted filled triangle), the K1061A mutant protein (inverted open triangle) or the K1086A mutant protein (filled square). The percentage of cells surviving each UV dose is shown. (**B**) The UV-induced mutagenesis of these same yeast strains. The number of *CAN1^S* to *can1^r* forward mutations per 10^7 surviving cells for each UV dose is shown.

strain (Figure 2A). Yeast expressing the wild-type pol ζ are more resistant to UV radiation than yeast lacking pol ζ. Yeast expressing either the R1057A or the K1086A mutant proteins were as sensitive to UV-radiation as the strain lacking pol ζ. Yeast expressing the K1061A mutant protein were less sensitive to UV-radiation than yeast lacking pol ζ, but were not as resistant as yeast expressing wild-type pol ζ.

We next assayed the mutant proteins for the ability to complement the defect in UV-induced mutagenesis of the *rev3Δ* yeast strain (Figure 2B). *CAN1^S* to *can1^r* forward mutations are readily observed in yeast expressing wild-type pol ζ upon exposure to UV radiation, but are greatly reduced in yeast lacking pol ζ. With the yeast cells expressing either the R1057A or K1086A mutant proteins, the incidence of UV-induced mutations was in between those of yeast expressing wild-type pol ζ and yeast lacking pol ζ. For the K1061A mutant protein, we observed nearly the same rate of UV-induced mutagenesis as with yeast expressing wild-type pol ζ.

Based on these genetic complementation assays, we conclude that the R1057A and K1086A mutant proteins are significantly more deficient in their *in vivo* functions than the K1061A mutant protein. Whatever defect the K1061A mutant protein might possess, it is able to function sufficiently *in vivo* to provide partial UV-resistance and nearly normal levels of UV-induced mutagenesis.

DNA polymerase activity of the pol ζ mutant proteins

We have purified wild-type pol ζ and the pol ζ mutant proteins and have examined their DNA polymerase activity *in vitro* using steady state kinetics. We first measured the ability of each of these proteins to catalyze

the incorporation of the correct dATP residue opposite template T following a normal, matched primer-terminal base pair (Figure 3 and Table 1). For wild-type pol ζ, the nucleotide was incorporated with a k_{cat} equal to $0.64\,min^{-1}$ and a K_m equal to $0.59\,\mu M$. For the R1057A mutant protein, the k_{cat} was $0.12\,min^{-1}$ and the K_m was $34\,\mu M$. This represents a 310-fold reduction in the catalytic efficiency (k_{cat}/K_m) for normal nucleotide incorporation. The K1086A mutant protein had a 500-fold decrease in catalytic efficiency relative to the wild-type protein. In contrast, the K1061A mutant protein had only a modest decrease (5.2-fold) in catalytic efficiency relative to the wild-type protein.

This major reduction in the efficiency of nucleotide incorporation by the R1057A and the K1086A mutant proteins *in vitro* is consistent with their inability to complement the UV-sensitivity of a yeast strain lacking pol ζ. The slight reduction observed with the K1061A mutant protein is also consistent with its ability to partially complement the UV-sensitivity of this strain. The greater severity of the substitutions of Arg-1057 and Lys-1086 relative to the substitution of Lys-1061 may be due to the fact that the residue in RB69 DNA polymerase corresponding to Lys-1061 (Lys-486) forms water-mediated bonds with the triphosphate moiety of the incoming dNTP rather than direct contacts with it as is the case with the residues corresponding to Arg-1057 and Lys-1086 (Arg-482 and Lys-560) (27).

Fidelity of the pol ζ mutant proteins

To ascertain the impact of these amino-acid substitutions on the fidelity of DNA synthesis, we examined the steady-state kinetics of the incorporation of the incorrect nucleotides opposite various template residues (Table 2 and Supplementary Table S1). On comparing the catalytic efficiencies for correct versus incorrect nucleotide incorporation for wild-type pol ζ, we found that the frequencies of misincorporation (f_{inc}) for these three incoming dNTPs ranged from 4.2×10^{-3} (1 in 240) for dGTP incorporation opposite T to 2.2×10^{-5} (1 in 45 000) for dCTP incorporation opposite C. These f_{inc} values are consistent with the previously reported range of f_{inc} values for pol ζ of 10^{-3}–10^{-5} (17).

We were unable to detect incorrect nucleotide incorporation with any of the three mutant proteins. Given the substantial decrease in catalytic efficiency for correct nucleotide incorporation for the R1057A and K1086A mutant proteins relative to the wild-type protein, this was not surprising. If these two proteins have f_{inc} values around 10^{-3}, then the catalytic efficiencies of incorrect nucleotide incorporation would be near the conservatively estimated detection limit of our assay, $2 \times 10^{-6}\,\mu M/min$. Thus, we are unable to draw conclusions about whether the fidelity of the R1057A and K1086A mutant proteins differs from that of the wild-type protein.

The inability to detect misincorporation with the K1061A mutant protein was surprising, because this protein has only a slight decrease in efficiency of correct nucleotide incorporation relative to wild-type pol ζ. Given the estimated detection limit of this assay, we conclude
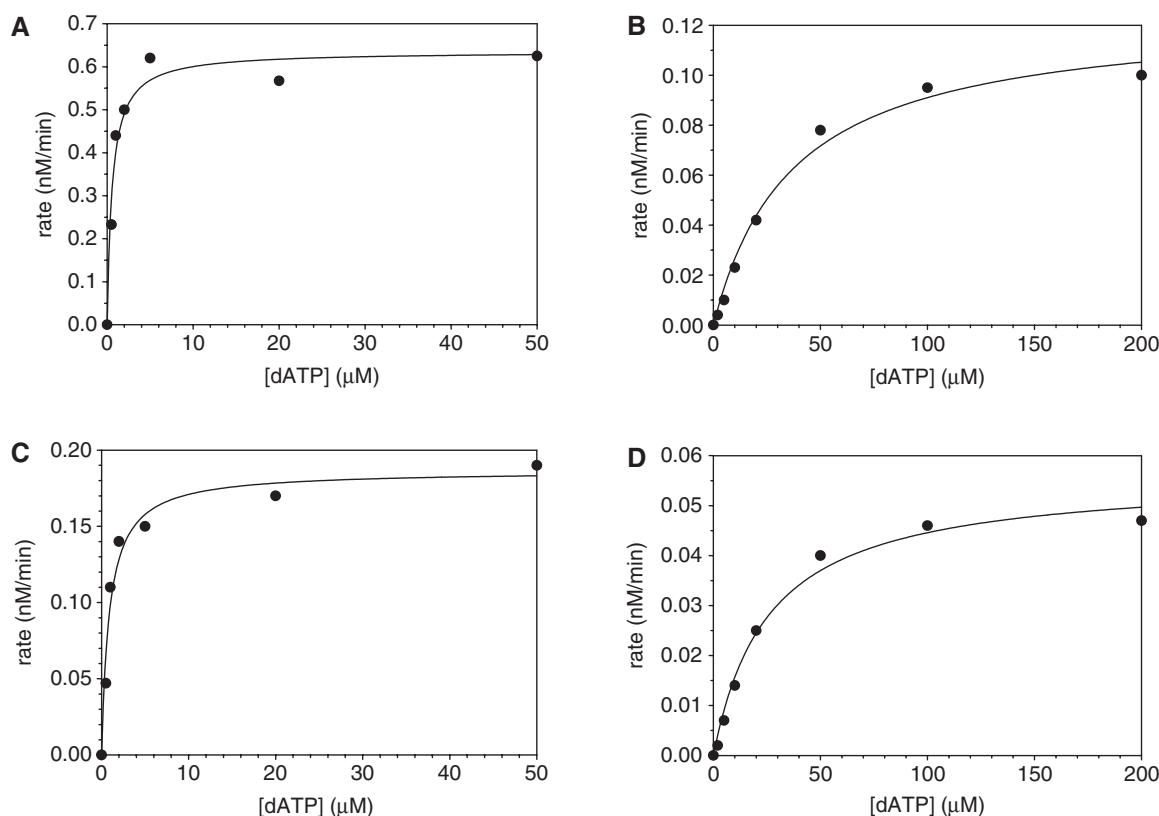
Figure 3. Steady-state kinetics of nucleotide incorporation by the pol ζ mutant proteins. The rate of nucleotide incorporation was graphed as a function of dATP concentration for (**A**) the wild-type pol ζ, (**B**) the R1057A mutant protein, (**C**) the K1061A mutant protein and (**D**) the K1086A mutant protein. The solid lines represent the best fits of the data to the Michaelis–Menten equation, and the k_{cat} and K_m steady-state parameters are given in Table 1.

Table 1. Nucleotide incorporation activity of pol ζ mutant proteins

Protein	dNTP·template	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM/min)	rel. eff.[a]
Wild-type	dATP·T	0.64 ± 0.003	0.59 ± 0.14	1.1	
R1057A	dATP·T	0.12 ± 0.01	34 ± 6	3.5×10^{-3}	310
K1061A	dATP·T	0.19 ± 0.01	0.91 ± 0.20	0.21	5.2
K1086A	dATP·T	0.057 ± 0.011	26 ± 4	2.2×10^{-3}	500

[a]The relative efficiency (rel. eff.) of nucleotide incorporation by a given mutant protein is equal to the k_{cat}/K_m for incorporation by the wild-type protein divided by the k_{cat}/K_m for incorporation by the mutant protein.

that the f_{inc} values for the K1061A mutant protein range from *less than* 4.8×10^{-5} (1 in 21 000) for template A to *less than* 2.5×10^{-6} (1 in 400 000) for template G. Thus, the fidelity of the K1061A mutant protein is greater than the fidelity of wild-type pol ζ. We are unable to determine how much the fidelity has improved in the mutant protein, but in the case of dGTP misincorporation opposite G, for example, the fidelity is increased by *at least* 120-fold (Table 2).

Extension abilities of the pol ζ mutant proteins

We next examined the steady-state kinetics of correct nucleotide incorporation following several primer-terminal mismatches to determine the impact of these amino-acid substitutions on the misextension ability of pol ζ (Table 3 and Supplementary Table S2). For wild-type pol ζ, the relative efficiency of extension from primer-terminal mismatches versus matches (f_{ext}^0) was approximately 0.49 (1 in 2.0) to 0.019 (1 in 53). These values are consistent with the previously reported range of values for pol ζ (17). The R1057A and K1086A mutant proteins had f_{ext}^0 values within the same range as those of the wild-type protein (data not shown). Interestingly, we found that the K1061A mutant protein had a decrease in the relative efficiency of mismatch extension compared to the wild-type protein. For example, the f_{ext}^0 for a A·G mismatch was 0.43 (1 in 2.3) for the wild-type protein and 0.053 (1 in 19) for the K1061A protein. In this case, the f_{ext}^0 of the mutant protein is about 8.1-fold lower than that of the wild-type protein (Table 3).

Table 2. Fidelity of the wild-type and K1061A mutant pol ζ proteins

dNTP·template	Wild-type		K1061A		Increase[b]
	k_{cat}/K_m (μM/min)	f_{inc}[a]	k_{cat}/K_m (μM/min)	f_{inc}[a]	
dGTP·G	5.2×10^{-4}	3.1×10^{-4}	$< 2 \times 10^{-6}$	$< 2.5 \times 10^{-6}$	> 120
dATP·G	2.8×10^{-4}	1.6×10^{-4}	$< 2 \times 10^{-6}$	$< 2.5 \times 10^{-6}$	> 64
dTTP·G	5.2×10^{-4}	3.1×10^{-4}	$< 2 \times 10^{-6}$	$< 2.5 \times 10^{-6}$	> 120
dCTP·G	1.7	NA	0.81	NA	NA
dGTP·A	2.4×10^{-4}	2.4×10^{-4}	$< 2 \times 10^{-6}$	$< 4.8 \times 10^{-5}$	> 5.0
dATP·A	1.9×10^{-4}	1.9×10^{-4}	$< 2 \times 10^{-6}$	$< 4.8 \times 10^{-5}$	> 4.0
dTTP·A	1.0	NA	0.042	NA	NA
dCTP·A	6.9×10^{-5}	6.9×10^{-5}	$< 2 \times 10^{-6}$	$< 4.8 \times 10^{-5}$	> 1.4
dGTP·T	4.6×10^{-3}	4.2×10^{-3}	$< 2 \times 10^{-6}$	$< 9.5 \times 10^{-6}$	> 440
dATP·T	1.1	NA	0.21	NA	NA
dTTP·T	4.3×10^{-4}	3.9×10^{-4}	$< 2 \times 10^{-6}$	$< 9.5 \times 10^{-6}$	> 41
dCTP·T	2.6×10^{-4}	2.4×10^{-4}	$< 2 \times 10^{-6}$	$< 9.5 \times 10^{-6}$	> 25
dGTP·C	5.5	NA	0.40	NA	NA
dATP·C	4.5×10^{-4}	8.2×10^{-5}	$< 2 \times 10^{-6}$	$< 5.0 \times 10^{-6}$	> 16
dTTP·C	1.1×10^{-3}	2.0×10^{-4}	$< 2 \times 10^{-6}$	$< 5.0 \times 10^{-6}$	> 40
dCTP·C	1.2×10^{-4}	2.2×10^{-5}	$< 2 \times 10^{-6}$	$< 5.0 \times 10^{-6}$	> 4.4

NA, not applicable.
[a]The frequency of misincorporation (f_{inc}) of a given incorrect dNTP is equal to the k_{cat}/K_m for incorporating the incorrect dNTP divided by the k_{cat}/K_m for incorporating the corresponding correct dNTP.
[b]The fold increase in fidelity for the K1061A mutant protein relative to the wild-type protein is equal to the f_{inc} for the wild-type protein divided by the f_{inc} for the mutant protein.

Table 3. Mismatch extension ability of the wild-type and K1061A mutant pol ζ proteins

Primer-terminal base pair[a]	Wild-type		K1061A		Decrease[c]
	k_{cat}/K_m (μM/min)	$f°_{ext}$[b]	k_{cat}/K_m (μM/min)	$f°_{ext}$[b]	
G·G	0.26	0.11	8.6×10^{-3}	0.062	1.8
A·G	1.0	0.43	7.4×10^{-3}	0.053	8.1
T·G	0.23	0.10	2.5×10^{-3}	0.018	5.6
C·G	2.3	NA	0.14	NA	NA
G·A	0.36	0.33	3.8×10^{-3}	0.019	17
A·A	0.44	0.40	7.8×10^{-3}	0.037	11
T·A	1.1	NA	0.21	NA	NA
C·A	0.40	0.36	1.9×10^{-3}	9.0×10^{-3}	40
G·T	0.56	0.081	9.3×10^{-3}	0.026	3.1
A·T	6.9	NA	0.36	NA	NA
T·T	0.13	0.019	2.4×10^{-3}	6.7×10^{-3}	2.8
C·T	0.23	0.033	4.1×10^{-3}	0.011	3.0
G·C	4.3	NA	0.19	NA	NA
A·C	2.1	0.49	0.018	0.095	5.2
T·C	1.0	0.23	6.9×10^{-3}	0.036	6.4
C·C	0.20	0.047	6.7×10^{-4}	3.5×10^{-3}	13

NA, not applicable.
[a]The primer-terminal base pairs are given as primer strand residue followed by template strand residue.
[b]The relative efficiency ($f°_{ext}$) for extending from a given primer-terminal mismatch is equal to the k_{cat}/K_m for extending from the mismatched primer-terminal base pair divided by the k_{cat}/K_m for extending from the corresponding matched primer terminal base pair.
[c]The fold decrease in mismatch extension ability of the K1061A mutant protein relative to the wild-type protein is equal to the $f°_{ext}$ for the wild-type protein divided by the $f°_{ext}$ for the mutant protein.

Studies of pol ζ mutant proteins with other substitutions at position 1061

Because the fidelity of the K1061A mutant protein was greater than that of wild-type pol ζ, we examined the effects of other amino-acid substitutions at this position by performing limited vertical scanning mutagenesis. We made three additional pol ζ mutant proteins with Arg, Leu or Glu substituted for Lys-1061 and measured the ability of each of these proteins to incorporate the correct incoming nucleotide (Supplementary Table S3). The K1061R mutant protein incorporates the correct nucleotide with approximately the same catalytic efficiency as does wild-type pol ζ. In contrast, the K1061L and K1061E mutant proteins incorporate the correct nucleotide with 180-fold and 270-fold lower catalytic efficiencies, respectively, compared to wild-type pol ζ.

We next measured the ability of these mutant proteins to incorporate incorrect incoming nucleotides. In the case of the K1061R mutant protein, incorporation of incorrect nucleotides was readily detectible, and the f_{inc} values were approximately the same as for the wild-type protein (Supplementary Table S3). Thus, the K1061R mutant protein does not incorporate nucleotides with a fidelity greater than that of wild-type pol ζ. In the case of the K1061L and K1061E mutant proteins, we were unable to detect incorrect nucleotide incorporation. Thus given the significant reduction in the catalytic efficiency of correct incorporation for these mutant proteins and given the detection limit of our assay, we are unable to draw conclusions about whether the fidelity of the K1061L and K1061E proteins differ from that of wild-type pol ζ.

DISCUSSION

To better understand the contribution of individual amino-acid residues to the enzymatic properties of pol ζ, we made amino-acid substitutions of three highly conserved residues in pol ζ (Arg-1057, Lys-1061 and Lys-1086) that correspond to residues in the bacteriophage RB69 DNA polymerase that contact the triphosphate
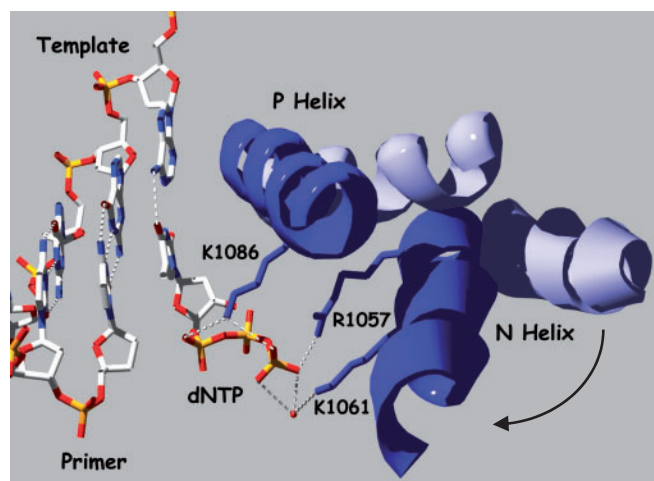
Figure 4. Model of the interactions of Arg-1057, Lys-1061 and Lys-1086 of pol ζ with the incoming dNTP. In the 'closed' conformation (dark blue), Arg-1057 from helix N makes a direct contact with the γ-phosphate of the incoming dNTP, and Lys-1061 from helix N makes a water-mediated contact with the γ-phosphate. Lys-1086 from helix P makes direct contacts with both the α-phosphate and γ-phosphate. This model is based on the structure of the RB69 DNA polymerase bound to both DNA and an incoming dNTP (27). Also shown are the relative positions of helices N and P in the 'open' conformation of RB69 DNA polymerase (light blue) (26).

moiety of the incoming nucleotide (Figure 4) (27). The residues corresponding to Arg-1057 and Lys-1061 (Arg-482 and Lys-486, respectively, in the RB69 DNA polymerase) are located in the N-helix of the fingers subdomain (26,27). The residue corresponding to Arg-1057 forms a hydrogen bond with a non-bridging oxygen on the γ-phosphate of the incoming dNTP, and the residue corresponding to Lys-1061 forms water-mediated bonds to non-bridging oxygens on the γ-phosphate. The residue corresponding to Lys-1086 (Lys-560 in the RB69 DNA polymerase) is located in the P helix of the fingers subdomain and forms hydrogen bonds to non-bridging oxygen atoms on the α-phosphate and the γ-phosphate of the incoming dNTP (26,27).

Substitutions of amino-acid residues in other class B family DNA polymerases corresponding to Arg-1057, Lys-1061 and Lys-1086 of pol ζ have been previously reported (38–41). In the bacteriophage RB69 DNA polymerase, substitution of the residue corresponding to Arg-1057 (Arg-482) resulted in a mutant protein with a 200-fold to 800-fold decrease in the efficiency (k_{cat}/K_m) of nucleotide incorporation activity relative to wild-type (40). Similarly, in the RB69 DNA polymerase, substitution of the residue corresponding to Lys-1061 (Lys-486) resulted in a 300-fold to 7500-fold decrease in the efficiency of nucleotide incorporation (40). Significantly decreased polymerase activity was also observed when the analogous residue in bacteriophage φ29 DNA polymerase (Lys-371) was substituted (41). Finally, substitution of the residues corresponding to Lys-1086 of pol ζ resulted in a 500-fold to 5600-fold decrease in the efficiency of nucleotide incorporation for RB69 polymerase (Lys-560) (40), a 60-fold decrease in the efficiency of nucleotide

incorporation for human pol α (Lys-950) (38), and no detectable DNA polymerase activity for the phage φ29 DNA polymerase (Lys-383) (39).

Our expectation, based on these prior studies, was that the amino-acid substitutions in pol ζ would result in mutant proteins with decreased DNA polymerase activity relative to wild-type pol ζ. With the R1057A and K1086A pol ζ mutant proteins, we obtained results similar to those reported for these other polymerases. The R1057A and K1086A mutant proteins had 310-fold and 500-fold reduced DNA polymerase activity relative to wild-type, respectively. Surprisingly, we obtained results with the K1061A mutant protein that significantly differed from those reported for these other DNA polymerases. We observed only a slight decrease in the efficiency of nucleotide incorporation (5.2-fold) relative to wild-type pol ζ.

Even more surprisingly, for the K1061A mutant pol ζ, we observed an increase in the fidelity of nucleotide incorporation of at least an order of magnitude. It should be noted that this increase in fidelity was almost entirely due to a decreased ability of the K1061A mutant protein to incorporate the incorrect nucleotide. A number of amino-acid substitutions have been found in other DNA polymerases that improve fidelity, but most of these do so by altering the balance between the DNA synthesis activity and the proofreading exonuclease activity (42,43). However, as pol ζ has no proofreading exonuclease function, this increase in fidelity must be due to increased selectivity of the nucleotide incorporation reaction itself. This phenomenon has been observed previously in only a few other polymerases. The most extensively studied of these is the low-fidelity HIV-1 reverse transcriptase, which also lacks a proofreading exonuclease function. In this case, fidelity is increased by amino-acid substitutions of Phe-61 (44) (contacting the single-stranded region of the template strand on the 5′ side of the template residue), Arg-72 (45) and Gln-151 (46,47) (contacting the incoming nucleotide), Asp-76 (48) and Arg-78 (49) (contacting the template residue), and Glu-89 (50) (contacting the duplex region of the template strand on the 3′ of the template residue). In addition, amino-acid substitutions were found that increase the fidelity of a proofreading-deficient form of *E. coli* DNA polymerase I, and these substitutions were distributed throughout the polymerase domain (51). Finally, an increase in fidelity was also observed with an amino-acid substitution at Tyr-52 (which may contact the incoming nucleotide via a water-mediated hydrogen bond) in TLS polymerase pol η (52).

In the case of amino-acid substitutions of Phe-61 of HIV-1 reverse transcriptase and Tyr-52 of pol η, only certain amino-acid substitutions at these positions resulted in mutant proteins with increased fidelity (44,52). For this reason, we examined other amino-acid substitutions of Lys-1061 of pol ζ. The highly conservative K1061R substitution resulted in a mutant protein with approximately the same activity and same fidelity as the wild-type protein. This suggests that the K1061R substitution is not disruptive enough to impact the fidelity of pol ζ. The moderately conservative K1061L and the

non-conservative K1061E substitutions resulted in proteins with substantially reduced polymerase activities, and we were therefore unable to measure the fidelity of these mutant proteins. It seems that only the moderately conservative K1061A substitution is disruptive enough to impact the fidelity of pol ζ, yet not disruptive enough to significantly reduce its catalytic activity. Without a high-resolution structure of pol ζ, it is difficult to draw firm conclusions about why certain substitutions of Lys-1061 dramatically effect the efficiency or the fidelity of nucleotide incorporation while other substitutions do not.

Another surprising aspect of the K1061A pol ζ mutant protein was that the substitution of an amino-acid residue contacting the triphosphate moiety of the incoming dNTP decreased the relative efficiency of misextension (f_{ext}^0) compare to wild-type pol ζ. Although this effect is much less than the effect on fidelity, this decrease in the relative efficiency of misextension was also due to a decreased ability of the K1061A mutant protein to extend from primer-terminal mismatches. Similar decreases in the relative efficiency of misextension were also observed with amino-acid substitutions of Arg-72 and Gln-151 of HIV-1 reverse transcriptase (45,46).

In summary, the K1061A mutant protein synthesizes DNA with a higher fidelity and extends from aberrant primer termini with somewhat lower efficiency than the wild-type protein. Thus, the K1061A mutant protein behaves more like a classical DNA polymerase in these respects than does wild-type pol ζ. These results show that in the wild-type protein, Lys-1061 plays an important role in accommodating the distorted DNA geometry of mismatches when they occur either at the nascent base pair or at the primer-terminal base pair.

Mechanistic and evolutionary implications

Classical DNA polymerases synthesize DNA with high fidelity because they possess a 'mismatch avoidance' function. Insight into this mismatch avoidance function has recently come from a set of high-resolution structures of human DNA polymerase β (32) and *Bacillus stearothermophilus* DNA polymerase I (33) bound to DNA substrates containing mismatches in their nascent base-pair binding pockets. These structures show that the bases of the nascent base pairs are in distinctly different positions when they are mismatched than when they are matched. For example, the bases of the mismatched C·A and C·T base pairs are not hydrogen bonded together in the active site of pol β; instead they are staggered and partially overlapping. Similarly, the template bases of the mismatched A·A, C·C and G·A base pairs are frayed out of the DNA helix into the 'pre-insertion site' of *B. stearothermophilus* pol I. The structural distortions of these mismatches within the nascent base-pair binding pockets of these enzymes are significantly greater than those of mismatched DNA free in solution. Thus, classical DNA polymerases avoid incorporating incorrect nucleotides by exaggerating the relatively minor structural distortions intrinsic to mismatches, and the resultant altered DNA conformations do not allow the

fingers subdomain to readily adopt the catalytically competent 'closed' state.

How might pol ζ differ mechanistically from these classical polymerases? One possibility is that during the evolutionary divergence of pol ζ from the classical polymerases in the class B family, this enzyme lost this mismatch avoidance function. If this were the case, then pol ζ would no longer exaggerate the minor structural distortions of mismatches, and the differences in DNA conformations between matched and mismatched base pairs when bound in the nascent base-pair binding pocket of pol ζ would not be as significant as when bound in this pocket of classical DNA polymerases. The discovery that a single amino-acid substitution in the active site of pol ζ can significantly increase its fidelity, however, argues against this possibility. If it were the case that pol ζ lost a mismatch avoidance function during its evolution, then the K1061A mutant protein would be a gain-of-function mutant protein. We believe it is highly unlikely that a single amino acid substitution could restore such an activity to pol ζ. Another, perhaps more interesting possibility is that during its evolution, pol ζ acquired a novel 'mismatch tolerance' function to help it overcome the barriers to efficient incorporation caused by the exaggerated structural distortions of the mismatched nascent base pair. In this case, the K1061A mutant protein would be a loss-of-function mutant protein; we believe it is far more likely that a single amino-acid substitution could disrupt a mismatch tolerance function of pol ζ.

Insight into how such a mismatch tolerance function might work comes from the finding that Lys-1061, which corresponds to an amino acid residue in helix N of the RB69 DNA polymerase that contacts the triphosphate moiety of the incoming dNTP, participates in this function. When a correct incoming dNTP binds to the RB69 DNA polymerases, helices N and P of the fingers subdomain rotate 60° into the 'closed' conformation (Figure 4) (27), in which conserved amino-acid residues make close contacts with the triphosphate moiety of the dNTP and help to position the α-phosphate, the 3′OH, and the metal ions for catalysis. A particularly intriguing possibility is that the mismatch tolerance function of pol ζ involves several active site residues, including Lys-1061, actively promoting the closing of the fingers subdomain when structural distortions are present in the active site.

While confirmation of this proposal awaits high-resolution structures of pol ζ, it does nicely explain the increased fidelity of the K1061A mutant protein. When pol ζ is bound to a correct incoming dNTP, there would be no structural distortions in the DNA, and the fingers subdomain would readily adopt the 'closed' conformational state. In this case, disengaging the tolerance function by substituting Ala for Lys-1061 would have little impact on enzymatic activity. However, when an incorrect incoming dNTP is bound, there would be a structural distortion in the DNA. Here, the tolerance function would be required for the fingers subdomain to adopt the 'closed' conformational state, and disengaging it would significantly reduce the efficiency of incorrect nucleotide incorporation. Interestingly, this notion also

nicely explains the somewhat decreased mismatch extension abilities of the K1061A mutant protein. When a mismatch is present in the primer-terminal position, structural distortions of the DNA around the nascent base pair also prevent the closing of the fingers subdomain. In the case of pol ζ, this tolerance function would help to facilitate closing of the fingers subdomain even when the primer-terminal base pair is mismatched. Thus, disrupting this tolerance function would also reduce the efficiency of mismatch extension by pol ζ.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

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