DNA polymerase β : analysis of the contributions of tyrosine-271 and asparagine-279 to substrate specificity and fidelity of DNA replication by pre-steady-state kinetics

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DNA polymerase β (pol β) from rat brain, overexpressed in *Escherichia coli*, was used as a model to study the factors responsible for substrate specificity [k_{pol} , $K_{\text{d}}(\text{app})$ and $k_{\text{pol}}/K_{\text{d}}(\text{app})$] and fidelity during DNA synthesis. The roles of two active-site residues, Asn-279 and Tyr-271, were examined by construction of N279A, N279Q, Y271A, Y271F and Y271S mutants followed by structural analyses by NMR and CD and functional analyses by pre-steady-state kinetics. The results are summarized as follows. (i) None of the two-dimensional NMR spectra of the mutants was significantly perturbed relative to that for wild-type pol β , suggesting that Tyr-271 and Asn-279 are not important for the global structure of the protein. (ii) CD analyses of guanidinium hydrochloride-induced denaturation showed that all mutants behaved similarly to the wild type in the free energy of denaturation, suggesting that Tyr-271 and Asn-279 are not critical for the conformational stability of pol β . (iii) The $K_{\text{d}}(\text{app})$ for the correct dNTP was lower than that for the incorrect dNTP by a factor of 10–30 in the case of wild-type pol β . Upon

mutation to give N279A and N279Q, the $K_{\text{d}}(\text{app})$ for the correct dNTP increased by a factor of 15–25. As a consequence, the $K_{\text{d}}(\text{app})$ values for the correct and incorrect nucleotides were similar for N279A and N279Q, suggesting that the main function of the side chain of Asn-279 is in discrimination between the binding of correct and incorrect dNTPs. (iv) In the case of the Y271A mutant, the fidelity and the catalytic efficiency $k_{\text{pol}}/K_{\text{d}}(\text{app})$ were little perturbed relative to the wild type. However, both the k_{pol} and $K_{\text{d}}(\text{app})$ values for dNTP were 4–8 times lower in the case of the Y271A mutant than the corresponding values for wild-type pol β . Since the chemical step may not be rate-limiting for wild-type pol β , the effect on k_{pol} could be quite significant if it is caused by a perturbation in the chemical step. (v) Pol β displayed the greatest specificity towards the G:C base pair, which is incorporated during base excision repair of G:U and G:T mispairs. This specificity was slightly enhanced for the Y271F mutant.

INTRODUCTION

Mammalian DNA polymerase β (pol β), with a molecular mass of 39 kDa [1], is one of the smallest DNA polymerases to have been characterized. The enzyme catalyses DNA-template-directed nucleotide transfer, and is thought to be involved mainly in DNA repair *in vivo*, particularly as a ‘gap-filler’ [2,3]. Under certain conditions pol β can also substitute for DNA polymerase I of *Escherichia coli* in the DNA synthesis of the lagging strand [4]. Pol β is an integral part of the base excision repair process [5–7]; it has been shown to participate in the repair of the G:U mispair by helping to remove dUMP and by synthesizing the correct pair G:C after dUMP has been removed.

This polymerase plays a key role in maintaining the integrity of the human genome; pol β mutations have been found in patients with certain types of colon and prostate cancers [8,9]. Detailed structure–function studies of the enzyme will provide valuable information pertaining to its mechanism, which may be helpful for understanding the molecular mechanisms that give rise to certain types of cancer.

The crystal structure of rat pol β has been reported [10], in which the enzyme is complexed with a duplex DNA and a nucleotide analogue, ddCTP. Although both the primary sequence and the general protein folding of rat pol β bear little resemblance to those of other well known DNA and RNA polymerases, including T7 and T4 phage DNA polymerases, the Klenow fragment of DNA polymerase I (KF) and T7 RNA polymerase, the overall shape of the enzyme is similar to those of

the other polymerases. They all consist of three main domains, commonly referred to as ‘palm’, ‘thumb’ and ‘fingers’, which form a wide U-shaped cleft that allows the enzyme to bind to a variety of nucleic acid substrates [10–14]. Also conserved is the three-dimensional arrangement of a triad of aspartate residues (Asp-190, Asp-192 and Asp-256) which are involved in the co-ordination of two bivalent metal ions responsible for catalysis [15].

Compared with other polymerases, pol β is highly error-prone [16–18], making mistakes in DNA synthesis an order of magnitude more often than exonuclease-deficient KF and 100 times more often than exonuclease-deficient T7 phage DNA polymerase. Pol β makes approximately the same number of mistakes as HIV reverse transcriptase, which is known for its high misincorporation frequency [19]. The factors that control substrate specificity and fidelity during DNA polymerization at the molecular level are still not well understood. Its relatively small size and the availability of the crystal structure of a pseudo-ternary complex make pol β an excellent system for investigating the molecular determinants and mechanisms involved in DNA synthesis, particularly in terms of dNTP specificity and fidelity.

As shown in Figure 1, the pseudo-ternary-complex crystal structure of pol β indicates that a number of active-site residues interact with the DNA and nucleotide substrates [10], among the most notable being Arg-283, Tyr-271 and Asn-279. Arg-283, which hydrogen-bonds directly to the template nucleotide, has recently been demonstrated by functional analyses to be a key factor in maintaining the fidelity of DNA synthesis [17,18,20]. In

Abbreviations used: GdnHCl, guanidinium hydrochloride; KF, Klenow fragment of DNA polymerase I; pol β , rat DNA polymerase β .

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Figure 1 Stereodiagram of the active site of pol β

Only three of the base pairs between the primer and template DNA are shown. The primer is terminated at the 3'-end by ddCMP (C7 of the primer strand). The incoming ddCTP molecule (DCT338) hydrogen-bonds with dGMP (G4) on the template. Two Mg^{2+} ions are shown co-ordinated by Asp-190, Asp-192 and Asp-256. Tyr-271 and Asn-279 are shown in the vicinity of the primer 3'-end and the incoming ddCTP. Arg-283 hydrogen-bonds to the template base (G4). Taken from the crystal structure solved by Pelletier et al. [10].

the present paper we assess the structural and functional significance of two other active-site residues, Tyr-271 (which is in the immediate vicinity of the incoming dNTP, and is thought to hydrogen-bond with the 3'-end primer base), and Asn-279 (which hydrogen-bonds directly to the incoming dNTP). Several mutants at these two positions were constructed and analysed by pre-steady-state studies in combination with structural analyses by NMR and CD.

While the present work was in progress, a paper by Beard et al. [20] was published, describing, among others, pol β mutants at positions 271 and 279. However, only steady-state kinetic analysis was reported in their work. Our use of pre-steady-state kinetics and conformational analysis by NMR have led to more detailed and revealing analyses of the structural and functional roles of these two residues.

MATERIALS AND METHODS

Materials

Substrate and mutagenic oligonucleotides were purchased from either Integrated DNA Technologies or Bio-Synthesis Inc. Substrate oligonucleotides were purified further by denaturing PAGE to homogeneity as described previously [17]. Concentrations of single-stranded oligonucleotides were measured by UV absorbance at 260 nm using a specific absorption coefficient of $0.03 \text{ ml} \cdot \mu\text{g}^{-1} \cdot \text{cm}^{-1}$ [21]. Ultra-pure dNTPs were purchased from Pharmacia-LKB. [γ - ^{32}P]dATP was obtained from ICN. Pol β cDNA was subcloned into M13mp19 phage DNA for mutagenesis at position 271, and into pET-17b (Novagen) for subsequent expression of pol β proteins. *E. coli* strains CJ236 and JM101 were used for mutagenesis at position 271. HB101 and BL21(DE3)pLysS *E. coli* strains were used for cloning and expression respectively of the proteins. A Chameleon double-stranded mutagenesis kit from Stratagene was used to create the mutants at position 279. All cell strains were obtained from Stratagene, except for BL21(DE3)pLysS (Novagen). All DNA-modifying enzymes were purchased from New England Biolabs, and all other reagents used were of the highest purity available commercially.

Mutagenesis, expression and purification of pol β mutants

Pol β mutants at position 271 were constructed in single-stranded M13mp19 phage DNA using the Kunkel mutagenesis method [22], and the mutants at position 279 were constructed in the

double-stranded pET-17b vector using a Chameleon kit from Stratagene. Pol β cDNA was subcloned into pET-17b downstream of the T7 promoter between the *Kpn*I and *Nde*I restriction sites. The desired mutations were confirmed by DNA sequencing.

Wild-type as well as mutant pol β proteins were expressed and purified as described by Werneburg et al. [17]. For some enzyme samples, the DNA-cellulose chromatography step in the previously described purification protocol was replaced by chromatography on heparin-Biogel (Bio-Rad). The proteins purified by the modified procedure behaved identically to the samples obtained by the original procedure, both electrophoretically and functionally. The purified proteins were judged to be > 95% pure as assessed by SDS/PAGE.

Enzyme activities were measured in a crude assay at ambient temperature with 50 mM Tris/HCl at pH 7.7 containing 120 mM KCl, 0.1 mM dithiothreitol, 2.5 mM $MgCl_2$, 40 $\mu\text{g}/\text{ml}$ poly(dA)/(dT)₁₂₋₁₈, 0.1 mM [^3H]dTTP (200 d.p.m./pmol) and 15% glycerol [23,24]. After adding 1 μl of the enzyme solution to 25 μl of the assay mixture, aliquots were removed as a function of time, quenched with 0.5 M EDTA and added to DEAE-cellulose filter paper discs (DE-81; Whatman). The discs were washed twice with 0.3 M sodium formate for 5 min and twice with ethanol for 5 min, and then air-dried and counted for radioactivity in 5 ml of scintillation cocktail to determine the amount of [^3H]dTTP incorporated as a function of time. The specific activity of the mutant enzymes as measured under these conditions varied from 10000 to 85000 units/mg, one unit of enzyme being defined as the amount incorporating 1 nmol of dTMP per h.

Protein concentrations were determined by absorbance at 280 nm using a molar absorption coefficient of $21\,200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [25]. The actual concentrations of mutant proteins Y271A and Y271S could be slightly different due to the replacement of a tyrosine residue. The enzyme concentrations given in the pre-steady-state kinetic analyses represent actual concentrations of enzyme active sites determined prior to the experiment by titration of the enzyme active sites in the reaction mixture [17]. It should be noted that, since $K_d(\text{app})$ and k_{pol} measurements were performed under the conditions of a large excess of enzyme over substrate DNA (usually 5:1), small deviations in the enzyme concentration should not affect the results.

Rapid and manual quench experiments

Rapid quench experiments were performed using a rapid quench apparatus designed by Johnson [26] and manufactured by KinTek Instruments Corp. The DNA primer (25-mer or 29-mer) was 5'-radiolabelled with [γ - ^{32}P]ATP (specific radioactivity 1000–

Table 1 DNA substrates for the kinetic assays

Substrate	Sequence
A	5' -GCCTCGCAGCCGTCCAACCAACATC 3' -CGGAGCGTCGGCAGGTTGGTTGAGTAGGAGCTAGGTTACGGCAGG
B	5' -GCCTCGCAGCCGTCCAACCAACATCACCT 3' -CGGAGCGTCGGCAGGTTGGTTGAGTAGGAGCTAGGTTACGGCAGG
C	5' -GCCTCGCAGCCGTCCAACCAACATC 3' -CGGAGCGTCGGCAGGTTGGTTGAGTAGGAGCTAGGTTACGGCAGG
D	5' -GCCTCGCAGCCGTCCAACCAACTCA 3' -CGGAGCGTCGGCAGGTTGGTTGAGTAGGAGCTAGGTTACGGCAGG

5000 d.p.m./pmol) using T4 polynucleotide kinase [21], and annealed to the 45-mer DNA template by incubating equimolar amounts of the oligonucleotides at 85 °C for 5 min, followed by gradual cooling (1.5 h) to room temperature. In a typical experiment, pol β (100–600 nM) was pre-equilibrated with annealed duplex DNA (200–400 nM) at 37 °C in a reaction buffer (50 mM Tris/HCl, pH 7.7, 50 mM KCl, 10% glycerol) containing 0.1 mg/ml BSA and 1 mM dithiothreitol. An aliquot of this solution (15 μ l) was then rapidly mixed in the rapid quench apparatus with an equal volume of dNTP substrate (1 μ M–8 mM) and 5 mM MgCl₂ in the reaction buffer. The reaction was terminated by addition of approx. 90 μ l of a 0.5 M EDTA solution, pH 8.0. All reactions were performed at 37 °C. For reaction times of 15 s and longer, manual quench experiments were performed. In this case, 15–20 μ l volumes of the same solutions were mixed manually in Eppendorf tubes, incubated at 37 °C for various periods of time (0.4–60 min) and quenched with 30–80 μ l of 0.5 M EDTA, pH 8.0.

The DNA template primer substrates used are shown in Table 1. Single-nucleotide incorporation, resulting in extension of the 25-mer or 29-mer DNA primer substrate to 26-mer or 30-mer respectively, was monitored by resolving the substrate and product on a denaturing 16% polyacrylamide gel and quantifying them using a BetaScope 603 blot analyser (Betagen). Either the formation of the product or the disappearance of the substrate was monitored as a function of time.

Conformational stability of pol β mutants

The conformational stabilities of the mutant proteins were assessed by CD as described by Werneburg et al. [17]. The proteins (0.2 mg/ml) were denatured with various concentrations of guanidinium hydrochloride (GdnHCl) in 5 mM borate (pH 8.0) containing 50 mM KCl at 22 °C, and the ellipticity at 222 nm was recorded. Experimental denaturation curves (ΔG_d against [GdnHCl]) were fitted to the equation $\Delta G_d = \Delta G_d^{H_2O} - m[GdnHCl]$ [27] to determine the free energy of unfolding ($\Delta G_d^{H_2O}$) and the slope (m), a constant related to the susceptibility of the enzyme to denaturation by the denaturant.

Two-dimensional NMR of pol β mutants

All NMR experiments were performed on a Bruker DMX-600 spectrometer. The chemical shifts were referenced to sodium [2,2,3,3-²H₄]3-(trimethylsilyl)propionate. Pol β samples for NMR were purified by a modified purification procedure [17]. The lyophilized samples were exchanged with ²H₂O for 10 min and lyophilized again. The final NMR samples contained 0.3 mM enzyme in ²H₂O with 40 mM [²H₁₁]Tris, 130 mM KCl, 0.04% NaN₃ and 1 mM dithiothreitol. The pH was adjusted to 7.5

without isotope correction, and the NMR spectra were recorded at 37 °C. A very small amount of precipitate was observed on the wall of the NMR tube at the end of the experiments (total duration was approx. 22 h). However, the one-dimensional spectra recorded before and after the two-dimensional experiments were identical, indicating that most of the sample was not denatured during the course of the experiment.

The NOESY experiments were performed with phase cycling, with a mixing time of 100 ms [28]. All spectra were obtained in the phase-sensitive mode using time-proportional phase incrementation and with water suppression using the 3–9–19 pulse sequence with gradients [29]. The spectral widths were 13 p.p.m. A total of 2048 $\times$ 416 matrices in the time domain were recorded and zero-filled to 2048 $\times$ 1024 matrices prior to multiplication by a Gaussian function (LB2 = –3; GB2 = 0.05) in the f2 dimension, a shifted sine bell (SSB = 8) in the f1 dimension, and Fourier transformation.

RESULTS

Expression and purification of mutant pol β proteins

All five mutant proteins (Y271A, Y271F, Y271S, N279A and N279Q) exhibited expression levels similar to that of the wild-type protein, expressed in *E. coli* strain BL21(DE3)pLysS [17]. The mutant proteins also behaved similarly to the wild-type throughout the purification procedure. Yields of 50–300 mg of electrophoretically homogeneous protein per 4 litres of cell culture were obtained.

Conformational analyses by NMR

The structural integrity of the mutant proteins was monitored by NMR and CD experiments. The two-dimensional NOESY spectra of wild-type pol β and of the N279A and Y271A mutants are shown in Figure 2 (aromatic–aliphatic cross-peaks) and Figure 3 (aromatic–aromatic cross-peaks). The low-field region (6–8 p.p.m.) of the spectra contains resonances from aromatic ring protons, histidine ring protons and some slow-exchanging amide protons, whereas the high-field region (0–5 p.p.m.) contains the resonances from aliphatic protons. The NOE cross-peaks between aromatic rings and aliphatic resonances, particularly those of methyl groups (0–1 p.p.m.), are good reporters of tertiary interactions. Some of the NOE cross-peaks between aromatic protons are also from inter-residue NOEs. Detailed examination of the NOESY spectra of the wild-type and mutant proteins indicates that most of the NOE cross-peaks in the wild-type spectra are retained in the mutant spectra. This suggests that the global tertiary structure of pol β is not perturbed by the introduced mutations.

Tyr-271 and Phe-272 are located in the active site of pol β , and may interact sterically with the ribose ring of the incoming nucleotide. The chemical shifts of Tyr-271 and Phe-272 and the inter-residue NOE cross-peaks between them could provide information about the local environment in the active site. It is thus useful to assign the aromatic resonances of these two residues, which was achieved by comparing the aromatic–aromatic region of the wild-type pol β spectrum with that of the Y271A spectrum (Figure 3). The aromatic resonances of Tyr-271 were assigned readily, since they were absent from the Y271A spectrum. Assignment of Phe-272 was based on the strong NOEs between Phe-272 and Tyr-271 in the wild-type spectrum and the disappearance of the NOE cross-peaks upon mutation of Tyr-271 to alanine. The assigned resonances are labelled in Figure 3. The Phe-272 peaks could not be identified in the spectrum of

Figure 2 Aromatic–aliphatic regions of two-dimensional NOESY spectra of wild-type pol β (A) and the mutants Y271A (B) and N279A (C)

The NMR samples contained 0.3 mM enzyme in $^2\text{H}_2\text{O}$ at pH 7.5. Mixing times were 100 ms, and spectra were recorded at 37 °C.

Figure 3 Aromatic–aromatic regions of two-dimensional NOESY spectra of wild-type pol β (A) and the mutants Y271A (B) and N279A (C)

The experimental conditions were as described in the legend to Figure 2. The Tyr-271 spin system is labelled Y and the Phe-272 spin system is labelled F. The inter-residue NOE peaks between Tyr-271 and Phe-272 are labelled n.

Y271A, but they were recovered at the original positions in the spectrum of Y271F (results not shown).

Conformational stability of the pol β mutants

The values for $\Delta G_d^{\text{H}_2\text{O}}$ of unfolding and the susceptibility constant m for the mutant proteins (Y271A, Y271F, Y271S and N279A) were found to be the same as the corresponding values for wild-type pol β (within experimental error) (Table 2), suggesting that the mutations at positions 271 and 279 did not alter the conformational stability of the protein.

The results of NMR and CD analyses, taken together, allowed us to conclude that the mutations did not lead to appreciable changes in the global conformation or the conformational stability of pol β . The integrity of the global conformation is a prerequisite for quantitative interpretation of the kinetic data in terms of the structure–function relationship, as described in the following sections.

DNA binding affinity

To determine whether mutations at positions 271 and 279 affect the ability of the enzyme to bind the DNA substrate, the K_d values for the Y271A·DNA and N279A·DNA complexes were determined as described previously [17,30]. At fixed enzyme concentrations, the DNA concentration was varied to generate a series of product against time curves that were fitted to the equation:

$$y = A(1 - e^{-k_{\text{obs}}t}) + mt \quad (1)$$

where A represents the burst amplitude, equivalent to the initial concentration of the enzyme·DNA complex, k_{obs} represents the observed burst rate constant and m is defined as the slope of the curve after the burst. The steady-state incorporation rate constant can be derived by dividing m by A . The obtained values of A were then plotted against the DNA concentration, and the data were fitted to a quadratic equation derived from the equilibrium equation to determine the K_d for the enzyme·DNA complex. The

Table 2 Free energy of GdnHCl-induced denaturation for wild-type pol β and mutants

Measurements were taken at 22 °C and pH 8.0. Values for the wild-type enzyme are taken from [17]. The error in $\Delta G_d^{H_2O}$ is estimated to be ± 2.1 kJ/mol. Note 1 kcal = 4.184 kJ.

Enzyme	$\Delta G_d^{H_2O}$ (kJ/mol)	m [kJ/(mol · M)]
Wild type	9.6	8.4
Y271A	12.1	8.4
Y271F	13.8	9.6
Y271S	13.0	9.6
N279A	11.3	11.7

K_d for the Y271A · DNA complex (66 ± 8 nM) was similar to that determined for wild-type pol β (49 ± 12 nM [17]). The K_d for the N279A · DNA complex was also similar (200 ± 80 nM). Thus the binding affinity of the enzyme for the DNA substrate was not

greatly perturbed upon replacement of either Tyr-271 or Asn-279.

Kinetic analyses and fidelity measurements

The mutant proteins were subjected to single-nucleotide incorporation assays in order to measure k_{pol} , $K_d(app)$ and fidelity (error frequency⁻¹), as described previously [17]. The results of the kinetic measurements were fitted to a burst equation:

$$y = A(1 - e^{-k_{obs}t}) \quad (2)$$

where A represents the burst amplitude and k_{obs} represents the observed burst rate constant [17,30]. To determine the catalytic rate constant (k_{pol}) and $K_d(app)$ values for the dNTPs, a series of product against time curves were obtained at various dNTP concentrations inclusive of $K_d(app)$ (Figures 4A and 4B). The enzyme concentration used was higher than the concentration of the duplex DNA substrate in order to eliminate the contribution of steady-state DNA synthesis to the results monitored. The

Figure 4 Single-turnover incorporation of dCTP or dATP opposite template dGMP by mutant Y271F

Reaction conditions and methods were as described in the Materials and methods section. The DNA substrate B (Table 1) was used in these experiments. (A), (B) Product formation as a function of time. The curves were fitted to eqn. (2) to give the observed rate constants (k_{obs}). (C), (D) Dependence of k_{obs} on dNTP concentration. The curves were fitted to eqn. (3) to give k_{pol} values of 3.30 ± 0.35 s⁻¹ and 0.016 ± 0.001 s⁻¹, and $K_d(app)$ values of 3.7 ± 1.2 μM and 166 ± 19 μM, for dCTP and dATP respectively.

Table 3 Kinetic parameters for wild-type pol β and the Asn-279 mutants

The substrates listed in Table 1 were used in these experiments. The concentrations of enzyme and DNA substrates were 430 nM and 85 nM respectively. The concentration of free MgCl_2 was 2.5 mM for wild type and 2.8 mM for the mutants. N/A, not applicable.

Base pair; template:dNTP	k_{pol} (s^{-1})	$K_{\text{d}}(\text{app})$ (mM)	$k_{\text{pol}}/K_{\text{d}}(\text{app})$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Fidelity
Wild type				
T:A	12 ± 2	66 ± 27	180 000	N/A
T:G	0.030 ± 0.001	650 ± 70	46	3 900
A:T	17 ± 1	41 ± 6	420 000	N/A
A:G	0.0079 ± 0.0002	980 ± 200	8	51 000
A:C	0.020 ± 0.001	200 ± 40	100	4200
G:C	3.4 ± 0.2	6.7 ± 0.6	510 000	N/A
G:A	0.014 ± 0.001	181 ± 36	78	6500
N279A				
T:A	16 ± 3	1400 ± 400	11 000	N/A
T:G	0.0053 ± 0.0008	980 ± 250	5.4	2 000
A:T	44 ± 10	1400 ± 600	31 000	N/A
A:G	0.010 ± 0.002	4300 ± 1300	2.3	14 000
A:C	0.011 ± 0.002	1800 ± 600	6.1	5100
N279Q				
T:A	14 ± 1	960 ± 130	15 000	N/A
T:G	0.0053 ± 0.0004	920 ± 120	5.8	2 600
A:T	14 ± 2	610 ± 120	23 000	N/A
A:G	0.0089 ± 0.0020	3600 ± 1400	2.5	9200
A:C	0.010 ± 0.001	2600 ± 500	3.8	6000

observed rate constants were plotted against the dNTP concentration (Figures 4C and 4D), and the data were fitted to the equation:

$$k_{\text{obs}} = k_{\text{pol}}[\text{dNTP}] / \{[\text{dNTP}] + K_{\text{d}}(\text{app})\} \quad (3)$$

to determine k_{pol} and $K_{\text{d}}(\text{app})$.

The fidelity of DNA replication by the enzymes is defined as the reciprocal of the misincorporation frequency, and is expressed as:

$$F = \{[k_{\text{pol}}/K_{\text{d}}(\text{app})]_{\text{i}} + [k_{\text{pol}}/K_{\text{d}}(\text{app})]_{\text{c}}\} / [k_{\text{pol}}/K_{\text{d}}(\text{app})]_{\text{i}} \quad (4)$$

where i and c denote incorrect and correct nucleotides respectively [31]. The kinetic parameters for correct (T:A, G:C and A:T) and incorrect (T:G, G:A, A:G and A:C) nucleotide incorporation are presented in Table 3 (wild-type pol β and Asn-279 mutants) and Table 4 (Tyr-271 mutants).

We used slightly different DNA substrates (Table 1) from those used in our previous work, which may explain the slight deviations in the kinetic constants for wild-type pol β from those reported previously [17,18]. In DNA substrate A (used for T:A and T:G base pairs), the three bases at the 3'-end of the primer and the corresponding bases of the template were rearranged in order to eliminate the possibility of partial melting/reannealing of the duplex. DNA substrate B was used to study G:C and G:A base pairs. DNA substrate D was used for A:G and A:T base pairs, but not for the A:C base pair, because it was found that in this case pol β was able to easily incorporate two correct (G:C) base pairs after the first incorrect base pair (A:C) was formed, which hindered interpretation of the experimental data. Therefore DNA substrate C was used for the A:C mispair.

Kinetics and fidelity of Asn-279 mutants

As shown in Table 3, the rates of catalysis at saturating dNTP concentrations (k_{pol}) were not significantly affected in mutants

Table 4 Kinetic parameters for the Tyr-271 pol β mutants

The concentrations of enzyme and DNA substrates were 500 nM and 100 nM respectively. The free MgCl_2 concentration was 2.5 mM. N/A, not applicable.

Base pair; template:dNTP	k_{pol} (s^{-1})	$K_{\text{d}}(\text{app})$ (mM)	$k_{\text{pol}}/K_{\text{d}}(\text{app})$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Fidelity
Y271A				
T:A	4.1 ± 0.2	14 ± 2	290 000	N/A
T:G	0.0085 ± 0.0003	100 ± 12	85	3400
A:T	2.31 ± 0.02	8.0 ± 0.2	290 000	N/A
A:G	0.0034 ± 0.0001	248 ± 73	14	21 000
A:C	0.023 ± 0.002	314 ± 86	73	4 000
G:C	0.58 ± 0.03	1.4 ± 0.2	415 000	N/A
G:A	0.0053 ± 0.0002	75 ± 7	71	5 800
Y271F				
T:A	4.0 ± 0.7	63 ± 25	63 000	N/A
T:G	0.0067 ± 0.0002	131 ± 13	51	1 200
A:T	2.8 ± 0.3	40 ± 12	70 000	N/A
A:G	0.0078 ± 0.0002	950 ± 40	8.2	8 500
A:C	0.013 ± 0.001	222 ± 32	58	1 200
G:C	3.30 ± 0.35	3.7 ± 1.2	890 000	N/A
G:A	0.016 ± 0.001	166 ± 19	93	9 600
Y271S				
T:A	1.1 ± 0.1	67 ± 26	16 500	N/A
T:G	0.0021 ± 0.0001	105 ± 16	20	825
A:T	2.1 ± 0.1	23 ± 3	90 000	N/A
A:G	0.0043 ± 0.0001	460 ± 10	9.2	9 800
A:C	0.015 ± 0.001	285 ± 28	52	1 700
G:C	1.0 ± 0.1	4.7 ± 0.5	210 000	N/A
G:A	0.0056 ± 0.0003	115 ± 17	49	4 300

N279A and N279Q. The largest change was a decrease in k_{pol} for the T:G mispair by a factor of 6, from 0.0300 s^{-1} for the wild type to 0.0053 s^{-1} for N279A and N279Q.

The main function of the side chain of Asn-279 seems to be in discriminating between the binding of correct and incorrect dNTPs. As shown in Table 3, the $K_{\text{d}}(\text{app})$ value for the correct dNTP was lower than that for the incorrect dNTP by a factor of 10–30 in the case of wild-type pol β . Upon mutation to N279A and N279Q, the $K_{\text{d}}(\text{app})$ values for the correct dNTPs were increased by a factor of 15–30, while those for the incorrect dNTPs increased by a smaller factor (approx. 0.5 for T:G, approx. 3 for A:G and 8–12 for A:C). As a consequence, the $K_{\text{d}}(\text{app})$ values were similar for the correct and incorrect nucleotides for N279A and N279Q, suggesting that the side chain of Asn-279 is necessary for selecting the correct nucleotides at the dNTP binding step.

The effects on fidelity were generally small: the largest observed was a factor of ~ 4 decrease in the fidelity of the A:G mispair for both N279A and N279Q. The fidelity in terms of the other base pairs studied was not significantly perturbed. Thus Asn-279 appears to play a minor role, if any, in maintaining the fidelity of pol β .

Kinetics and fidelity of Tyr-271 mutants

As shown in Table 4, the catalytic efficiency [$k_{\text{pol}}/K_{\text{d}}(\text{app})$] and fidelity of Y271A were different from the corresponding values for wild-type pol β only within a factor of 2. These data would seem to suggest that Tyr-271 does not play any significant catalytic role. However, detailed analysis showed that both k_{pol} and $K_{\text{d}}(\text{app})$ values decreased by a factor of up to 7 for both correct and incorrect base pairs upon replacement of the tyrosine residue by alanine. The only base pair not affected was A:C.

Although the effects on k_{pol} and $K_{\text{d}}(\text{app})$ were modest at best and also cancelled each other out, it is interesting to observe an increase in the dNTP binding affinity [a decrease in $K_{\text{d}}(\text{app})$] in the Y271A mutant.

When the phenyl ring was re-introduced at this position (Y271F), the binding affinities for all of the base pairs except T:G were decreased back to close to wild-type levels. The effect on k_{pol} , however, was not restored for the Y271F mutant, except for G:C and G:A (which is addressed below), which led to somewhat lower fidelity for T:G, A:G and A:C mismatches. The mutant Y271S exhibited intermediate changes in kinetic parameters compared with those of Y271A and Y271F.

G:C specificity

Pol β has been shown to be involved in the repair of G:T [32] and G:U [6] mispairs, specifically by helping to remove dUMP [5] and incorporating dCMP opposite dGMP [7]. Of the three correct base pairs measured in the present study, pol β had the greatest affinity for the G:C base pair [$K_{\text{d}}(\text{app})$ 6.7 μM , which is 6–10 times lower than those for the correct A:T and T:A base pairs). Similar results have also been reported by Ahn et al. [18] using slightly different DNA substrates. Upon mutation of Tyr-271 to phenylalanine, the specificity constants [$k_{\text{pol}}/K_{\text{d}}(\text{app})$] for the A:T and T:A base pairs decreased by factors of 4–5, while that of G:C increased by a factor of 2, resulting in an even greater specificity for the G:C base pair relative to A:T and T:A.

DISCUSSION

The pre-steady-state kinetic experiments coupled with NMR and CD analyses of site-specific mutants of pol β presented here have given new insight into the molecular basis of DNA replication fidelity at the polymerase active site. The NMR and CD analyses indicated that the side chains of Tyr-271 and Asn-279 are not important in maintaining the three-dimensional structure or conformational stability of pol β . The results of DNA binding experiments indicated that the mutants of Tyr-271 and Asn-279 retain their DNA binding affinity. The fidelity also remains practically unchanged in the mutants. However, the two residues appear to participate in the catalytic function of pol β by affecting k_{pol} and $K_{\text{d}}(\text{app})$. The interpretations and implications of the results are elaborated below.

Asn-279 helps to discriminate against binding of the incorrect dNTP

It has been proposed by Wong et al. [31] that selection against incorrect base pairs by T7 phage DNA polymerase involves two main steps: initial binding discrimination and kinetic discrimination at the conformational change step preceding the chemical reaction. Evidence for rate-limiting conformational changes in the mechanisms of polymerases, including pol β , have been observed [17,20,33–35]. The relative contributions of these two steps to fidelity vary for each polymerase.

The results obtained from pre-steady-state kinetic experiments with the N279A and N279Q mutants suggest that the main role of Asn-279 is to discriminate between correct and incorrect nucleotides at the dNTP binding step. Asn-279 is positioned appropriately for hydrogen-bonding with the incoming correct nucleotide [10], and presumably provides stabilization of the ternary complex in the case of correct base pairs. The ability of the enzyme to distinguish between correct and incorrect nucleotides at this step was completely abolished upon mutation of

Asn-279 to alanine. Mutation to glutamine at position 279 restored some of the enzyme's ability to select against incorrect nucleotides, but not to the level exhibited by the wild-type enzyme.

While the two proteins with mutations at Asn-279 were less capable of discriminating between the correct and incorrect base pairs at the binding step, the fidelity in terms of two of the three mispairs measured decreased only slightly. However, the effect of the Y279A mutation on the overall fidelity of pol β -mediated DNA synthesis *in vivo* could be more pronounced due to subsaturating dNTP concentrations.

Tyr-271 mutations affect both k_{pol} and $K_{\text{d}}(\text{app})$

The crystal structure of a pseudo-ternary complex of pol β [10] implies that Tyr-271 places several constraints on the positions of the primer DNA strand and the dNTP substrate (Figure 1). The phenolic ring of Tyr-271 is positioned snugly between the primer 3'-end base and the base of the incoming nucleotide, and is thought to be involved in hydrogen-bonding with the base of the primer oligonucleotide, breaking up organized water clusters in the minor groove of the bound DNA duplex, as well as contributing to the steric environment at this particular site [10]. The strategic placement of Tyr-271 in the structure of the enzyme makes it a good candidate for a potential regulator of substrate specificity, as even small changes at this site could have profound effects on the binding of the incoming nucleotide. Although pol β exhibits little primary sequence homology to any of the characterized DNA polymerases, the position of Tyr-271 in the structure of the enzyme may be analogous to that of a highly conserved tyrosine residue in the active sites of several polymerases [36,37]. It has been shown previously [33] that residue Tyr-766 in KF, a possible functional analogue of Tyr-271 in pol β , plays an important role in DNA replication fidelity.

As indicated by the data in Table 4, the fidelity of the Tyr-271 mutants was only slightly altered from that of the wild-type pol β shown in Table 3. However, detailed analyses of the data suggested that both the k_{pol} and $K_{\text{d}}(\text{app})$ values were perturbed somewhat in the Tyr-271 mutants. Mutation of Tyr-271 to alanine led to small decreases in k_{pol} . The effect was more pronounced in the case of correct pairs, where k_{pol} was decreased by a factor of 3–8. The decreases in k_{pol} values were more pronounced for Y271S than for Y271A, while those for Y271F were less pronounced. Since k_{pol} is a reflection of a combination of the chemical step and a putative conformational change induced by dNTP binding [17], the decrease in k_{pol} could have been caused by a decrease in a rate of the chemical step, a rate of the putative conformational step, or both. We have now identified the conformational change and showed that it is the rate-limiting step in catalysis by pol β (X. Zhong, unpublished work). Thus, if the decreases in k_{pol} were caused mainly by a perturbation in the chemical step, the perturbation would in fact be substantially greater than that reflected in the observed effects on k_{pol} .

In addition to the effect on k_{pol} , the mutation of Tyr-271 to alanine also caused a decrease in the $K_{\text{d}}(\text{app})$ for correct nucleotides by a factor of approx. 5; the decreases were smaller for incorrect nucleotides. Although a factor of 5 is a small effect, it is unusual for the $K_{\text{d}}(\text{app})$ values of a mutant to decrease. Pol β , which binds correct nucleotides more weakly than KF [17,18,33], T7 phage DNA polymerase [30] and HIV reverse transcriptase [19], acquired greater affinity for the three correct base pairs measured in the present work upon mutation of Tyr-271 to alanine. A similar effect was found for KF when Tyr-766 was replaced by serine [33].

The decreases in the $K_d(\text{app})$ values could be due to a steric effect on dNTP binding. By effectively eliminating the steric bulk of the amino acid side chain at this position, a larger binding pocket could be created in the active site of the protein, presumably better accommodating correct base pairs and some incorrect base pairs with distorted ('wobble') molecular geometry [38,39], such as A:G, G:A or T:G. The fact that the $K_d(\text{app})$ values of the Y271F mutant were generally much closer to the wild-type values than were those of Y271A supports the importance of steric interactions with respect to the nucleotide binding properties of the enzyme.

Asymmetry of misincorporation efficiencies

It is worth noting that the misincorporation efficiencies for the A:G and G:A mispairs were found to be asymmetrical for the Tyr-271 mutants and wild-type pol β . This was also found to be the case for KF [33], and may prove to be universal due to the asymmetric nature of the enzyme binding sites, as well as the intrinsic asymmetry of base pairs.

G:C specificity

The fact that DNA polymerase I-deficient *E. coli* can be 'rescued' by pol β suggests that pol β can perform the same functions as polymerase I, including DNA repair and synthesis of the lagging strand during replication of the *E. coli* genome [4]. Perhaps the most important function of pol β in mammals is the replacement of G:T and G:U mispairs before they give rise to A:T transversions in the genome [6,32]. Consistent with the idea that the main function of pol β pertains to base excision repair [7], the results of pre-steady-state kinetic analyses presented in this paper and those described by Ahn et al. [18] using slightly different substrates indicate that pol β has greater specificity for the G:C base pair than for the other correct pairs on regular duplex DNA substrates. The biological relevance of this specificity remains to be established; however, it is interesting that Y271F appears to be more specific for the G:C base pair than does the wild-type enzyme. While the ratio $k_{\text{pol}}/K_d(\text{app})$ was decreased for T:A and A:T base pairs in the Y271F mutant, it was increased slightly for the G:C base pair. These results suggest that position 271 is important for the specificity of the enzyme towards the G:C base pair.

Comparison with the previous results

The approaches described in the present paper are different from those used by Beard et al. [20], who also reported studies on Tyr-271 and Asn-279 mutants of pol β . They performed steady-state kinetics on a homopolymeric [poly(dA/dT)] DNA template/primer, while we performed pre-steady-state kinetics on DNA heteroduplexes. Our direct measurements of misincorporation efficiencies in an isolated *in vitro* system allowed us to detect all incorporation events that occurred, unlike the short gap reversion assay used in [20], which fails to take phenotypically silent mutagenic events into consideration and is dependent on the transformation efficiency. Furthermore, investigation of the structural properties of Tyr-271 and Asn-279 mutants by NMR and CD allowed us to interpret the kinetic data with a greater degree of confidence, as the enzymes were clearly shown to be unaffected by the introduced substitutions.

Our results agree with those of Beard et al. [20] in that mutations of Tyr-271 or Asn-279 led to only small changes in fidelity. However, it would be incorrect to conclude that the two residues do not play a functional role in catalysis by pol β . The determination of k_{pol} and $K_d(\text{app})$ values by pre-steady-state

kinetics allowed us to conclude that the side chain of Asn-279 helps to discriminate against incorrect dNTPs at the nucleotide binding step. The side chain of Tyr-271 exerts a steric hindrance to dNTP binding [$K_d(\text{app})$ values decrease in Y271A], but it probably helps to control the dNTP specificity at the chemical step and/or the putative conformational step. Furthermore, we have identified increased G:C specificity for the mutant Y271F.

As a closing remark, we emphasize that even the detailed analyses presented here may not be sufficient. Since the effects of mutations of Tyr-271 and Asn-279 on k_{pol} and $K_d(\text{app})$ are generally small (a factor of 10 or less), and since the chemical step and the conformational step have not been dissected, the quantitative interpretation should be treated with caution. Furthermore, only seven of the 16 possible base pairs have been analysed in the present work. A complete analysis of all possible base pairs, as in the case of the R283A mutant [18], and a detailed dissection of the kinetic steps will be needed in order to further enhance our understanding of structure-function relationships in pol β .

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