DNA polymerase β: effects of gapped DNA substrates on dNTP specificity, fidelity, processivity and conformational changes[1]

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Pre-steady-state kinetic analysis was used to compare the catalytic properties of DNA polymerase β (Pol β) for single-base gap-filling and regular duplex DNA synthesis. The rate of polymerization (k_{pol}) and the apparent equilibrium dissociation constant of dNTP (K_d) were determined with single-nucleotide gapped DNA substrates for all four possible correct base pairs and twelve possible incorrect base pairs, and the results were compared with those obtained previously with non-gapped primer/template duplex DNA substrates. For correct dNTP incorporation, the use of single-nucleotide gapped DNA led to significant decreases in the K_d of dNTP. Although k_{pol} was little affected, the catalytic efficiency k_{pol}/K_d increased significantly owing to the decreases in K_d. In contrast, for incorrect dNTP incorporation, the use of single-nucleotide gapped DNA substrates did not affect the K_d of dNTP appreciably but caused the k_{pol} (and thus k_{pol}/K_d) for incorrect dNTP incorporation to increase. As a consequence the fidelity of Pol β was not significantly affected by the use of single-nucleotide gapped DNA substrates. In addition we show that under processive polymerization conditions the processivity of Pol β increases in the gap-filling synthesis owing to a decreased rate of DNA dissociation. Finally, with a single-nucleotide gapped DNA substrate the rate-limiting conformational change step before chemistry was also observed. However, the preceding fast conformational change observed with duplex DNA substrates was not clearly detected. A possible cause is that in the complex with the gapped DNA, the 8 kDa N-terminal domain of Pol β already exists in a closed conformation. This interpretation was supported by tryptic digestion experiments.

INTRODUCTION

Genomic DNA is constantly damaged by various physical and chemical agents [1,2]. Repair of the damaged DNA is conducted via several complex mechanisms such as nucleotide excision [3], base excision [1] and double-strand break repair by recombination [4]. The importance of DNA repair has been recognized since the relation between defective DNA repair and various forms of cancers was revealed. For example, hereditary non-polyposis colorectal cancer can be caused by germline mutation of the mismatch repair genes [5–9].

DNA polymerase β (Pol β) is one of the smallest (39 kDa) $5' \rightarrow 3'$ DNA polymerases known. It is also one of the simplest DNA polymerases because it lacks other intrinsic exonuclease or endonuclease activities [10]. Numerous studies have been conducted to show that Pol β is involved in single-nucleotide gap-filling synthesis as a part of base excision repair. The repair of G:U and G:T mispair was mediated by Pol β, based on studies of mammalian crude cell extract *in vitro* [11–13]. Treatment of cells with DNA-alkylating agents has been shown to up-regulate the expression level of Pol β [14,15]. The most convincing and direct study *in vivo* with mouse cell lines homozygous for a deletion mutation in the Pol β gene was reported by Sobol et al. [16]. The cell lines showed defectiveness in uracil-initiated base excision repair and sensitivity to monofunctional DNA-alkylating agents, which were rescued on transfection with a Pol β minitransgene. Another piece of evidence for the role of Pol β in base excision repair is that the N-terminal 8 kDa domain of Pol β can function to remove the deoxyribose phosphate moiety from incised apurinic:apyrimidinic sites [17].

Recently we reported the use of pre-steady-state kinetics to determine the substrate specificity and fidelity of Pol β (from rat brain, overexpressed in *Escherichia coli*) and a few active-site residue mutants [18–20]. We also used stopped-flow fluorescence to observe fast and slow conformational changes occurring before the chemical step [21]. In these studies we used primer/template DNA duplex as a substrate because we intended to focus on Pol β as a model of other polymerases. In the present work we shift our focus to the mechanism of Pol β in DNA repair by examining the effects of gapped DNA substrates on the rate of dNTP incorporation (k_{pol}), the apparent equilibrium dissociation constant of dNTP (K_d), the fidelity, the k_{off} of DNA, the processivity and the conformational changes.

MATERIALS AND METHODS

Materials

Ultra-pure dNTPs were purchased from Pharmacia, and [γ-^{32}P]ATP and [α-^{32}P]dNTP were from ICN Biomedicals. BSA free of nuclease and protease activities was obtained from Boehringer Mannheim. T4 polynucleotide kinase was purchased from New England Biolabs. Sequenase 2.0 or T7 Pol (exo$^-$) was purchased from United States Biochemical. DE-81 filters were obtained from Whatman. G-25 microspin columns were purchased from Pharmacia Biotech. All other reagents were of the highest purity available commercially. Pol β was purified as described [22] from an overexpressing *E. coli* system, BL21(DE3)[plysS, pET17-Pol β] [20]. The enzyme was estimated to be more than 98% homogeneous on the basis of SDS/PAGE analysis developed

Abbreviations used: Ã, 2-aminopurine nucleotide analogue; ddAMP, dideoxy AMP; DTT, dithiothreitol; Pol β, rat DNA polymerase β.

[1] This is no. 6 in the series; no. 5 is Zhong, X., Patel, S. S. and Tsai, M.-D. (1998) J. Am. Chem. Soc. **120**, 235–236.

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A. 25A/45TX

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5'-GCCTCGCAGCCGTCCAACCAACTCA-3'
3'-CGGAGCGTCGGCAGGTTGGTTGAGTXYGAGCTAGGTTACGGCAGG-5'
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B. 25A-19/45TX

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5'-GCCTCGCAGCCGTCCAACCAACTCA ZCTCGATCCAATGCCGTCC-3'
3'-CGGAGCGTCGGCAGGTTGGTTGAGTXYGAGCTAGGTTACGGCAGG-5'
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25A-18/45TA

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5'-GCCTCGCAGCCGTCCAACCAACTCA  CTCGATCCAATGCCGTCC-3'
3'-CGGAGCGTCGGCAGGTTGGTTGAGTAGGAGCTAGGTTACGGCAGG-5'
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25A-21/50TA

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5'-GCCTCGCAGCCGTCCAACCAACTCA     CGATCCAATGCCGTCCTAGCG-3'
3'-CGGAGCGTCGGCAGGTTGGTTGAGTAGGAGCTAGGTTACGGCAGGATCGC-5'
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25A-18/50TA

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5'-GCCTCGCAGCCGTCCAACCAACTCA        TCCAATGCCGTCCTAGCG-3'
3'-CGGAGCGTCGGCAGGTTGGTTGAGTAGGAGCTAGGTTACGGCAGGATCGC-5'
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C. 20A-15/36TÃ

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5'-GCCTCGCAGCCGTCCAACCA AGTCACCTCAATCCA-3'
3'-CGGAGCGTCGGCAGGTTGGTÃTCAGTGGAGTTAGGT-5'
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19C-15/36GTÃ

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5'-GCCTCGCAGCCGTCCAACC  AGTCACCTCAATCCA-3'
3'-CGGAGCGTCGGCAGGTTGGTÃTCAGTGGAGTTAGGT-5'
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Figure 1 Sequences of primer/template and primer-downstream oligonucleotide/template DNA

The numbers in the names represent the lengths of the oligonucleotides. (**A**) Primer/template (regular duplex DNA) substrate; the bases XY are AG for 25A/45TA, TG for 25A/45TT, CG for 25A/45TC and GC for 25A/45TG. (**B**) Primer-downstream oligonucleotide/template (gapped DNA) substrate. 25A-19/45TX are single-nucleotide gapped DNA substrates; the bases XY and Z are AG and C, for 25A-19/45TA; TG and C for 25A-19/45TT; CG and C for 25A-19/45TC; and GC and G for 25A-19/45TG. 25A-18/45TA, 25A-21/50TA and 25A-18/50TA are two, four and seven-nucleotide gapped DNA substrates respectively. Downstream oligonucleotides are always 5′ phosphorylated. (**C**) Gapped DNA substrates containing 2-aminopurine (Ã).

by the silver staining method [23]. The enzyme concentration was determined by using a molar absorption coefficient of 21200 $M^{-1} \cdot cm^{-1}$ at 280 nm [24].

DNA substrates

Custom-synthesized DNA oligomers were purchased from IDT (Coralville, IA, U.S.A.). The DNA oligomers were further purified by electrophoresis in 16 % (w/v) polyacrylamide/7 M urea gels. Appropriate sizes of regular duplex and gapped DNA substrates were prepared as described by Ahn et al. [18]. The sequences of the DNA substrates used in this study are shown in Figure 1.

The exact concentration of the regular duplex (or gapped) DNA was determined by incorporation of the correct [α-^{32}P]dNTP by using Sequenase 2.0. In a total volume of 75 μl, 20 nM DNA, 1 μM appropriate [α-^{32}P]dNTP (25 Ci/mmol), 2 units of Sequenase 2.0, 20 mM $MgCl_2$, 50 mM NaCl and 40 mM Tris/HCl, pH 7.5, were incubated at 25 °C. After 1–3 min, 20 μl aliquots were withdrawn and quenched with 10 μl of 0.5 M EDTA, pH 8.0. The amount of radioactivity incorporated into DNA substrate was determined by the DE81 filter binding assay [25,26]. The radioactivity was the same for three different reaction times. The potential problem with this method is the possible misincorporation of [α-^{32}P]dNMP after the first correct nucleotide incorporation: this could give a false positive concentration of DNA. To make sure that this does not happen, one incorrect [α-^{32}P]dNTP was incubated with the enzyme and DNA substrate, and radioactivity was counted by performing the filter binding assay. The radioactivity of the filter was close to the background level (negative control), ensuring that the misincorporation of [α-^{32}P]dNMP was not a significant factor in determining the concentration of DNA.

For the chemical quench experiment the DNA substrate was 5′-end-labelled with ^{32}P by incubating with T4 polynucleotide kinase and [γ-^{32}P]ATP (4500 Ci/mmol) by following the manufacturer's protocol. The 5′-radiolabelled DNA was separated from unreacted [γ-^{32}P]ATP on a G-25 microspin column. The labelled DNA was mixed with an approx. 100-fold molar excess of unlabelled DNA and the T4 polynucleotide kinase was inactivated at 65 °C for 15 min. The solution was cooled slowly to room temperature.

Chemical quench experiments and product analysis

A rapid quench instrument (KinTek Instrument Corp., State College, PA, U.S.A.) was used for reaction times ranging from 5 ms to 20 s. Pol β or T7 Pol (exo$^-$) were preincubated with a DNA substrate before rapid mixing with dNTP and $MgCl_2$ to begin the reaction, which was quenched with 0.3 M EDTA (final concentration). For reaction times greater than 20 s, 20 μl aliquots of the reaction mixture (total volume 140 μl) were removed and mixed with equal volumes of 0.6 M EDTA, pH 8.0, manually after certain time intervals. The typical experiment was performed at 37 °C in 50 mM Tris/HCl, pH 7.7, containing 50 mM KCl, 10 % (v/v) glycerol, 0.2 mg/ml BSA, 1 mM dithiothreitol (DTT), 2.5 mM $MgCl_2$ (free). A sample of quenched reaction (20 μl) was mixed with an equal volume of gel loading buffer [27], denatured at 85 °C for 5 min and run on a 16 % (w/v) polyacrylamide/7 M urea gel. The disappearance of substrate and the formation of product were monitored with a β-scanner (Betagen) as described by Werneburg et al. [20].

Measurement of k_{off} for DNA by competition experiment

The dissociation constant for DNA was measured with a fast-quench apparatus as follows. The enzyme–DNA complex solution [consisting of 150 nM 25A/45TT duplex DNA (^{32}P-labelled at the 5′ end of the primer), 200 nM Pol β, 2.5 mM $MgCl_2$, 2.5 mM DTT and 0.25 mg/ml BSA in 50 mM Tris/HCl, pH 7.7, containing 50 mM KCl and 10 % (v/v) glycerol] was mixed in a fast-quench apparatus with the 'trap' DNA solution [150 μM unlabelled duplex 12/24-mer DNA in 50 mM Tris/HCl (pH 7.7)/50 mM KCl/2.5 mM $MgCl_2$/10 % (v/v) glycerol], and incubated for various periods (0.02–12 s). The reaction was 'quenched' with 200 μM dATP in 50 mM Tris/HCl (pH 7.7)/50 mM KCl/2.5 mM $MgCl_2$/10 % (v/v) glycerol, incubated for a fixed time of 10 s, then quenched with 0.5 M EDTA. Products were separated on a denaturing polyacrylamide gel as described above; amount of 26-mer formed was quantified and fitted to the following equation to determine the k_{off} for DNA:

$$[\text{26-mer}] = A \exp(-k_{off} t) + m \quad (1)$$

Stopped-flow fluorescence assay

The experiments were performed with a KinTek Stopped-Flow apparatus. The excitation wavelength was 290 nm and the emission was monitored with a 340 nm bandpass filter (Corion). Typical assay conditions were as follows. In one syringe, 1.2 μM rat DNA polymerase β was incubated with 0.6 μM 20A-15/36TÃ DNA substrate (where Ã represents 2-amino purine) and 5 mM $MgCl_2$. The second syringe contained 200 μM dTTP and 5 mM $MgCl_2$. The reaction was incubated by mixing 80 μl of solution from each syringe; the time course of the fluorescence signal was monitored during the reaction. Multiple experiments were

performed and the results were averaged (9–16 runs) to maximize the signal-to-noise ratio. To prepare $20A_{dd}$-15/36TÃ, a DNA substrate that contains dideoxyAMP (ddAMP) at the 3′-end of the primer, 1.2 μM Pol β, 0.6 μM 19C-15/36GTÃ, 2 μM ddATP and 5 mM $MgCl_2$ were preincubated in a syringe to allow ddAMP to be incorporated into the primer. The stopped-flow reaction was then initiated by adding 200 μM dTTP/5 mM $MgCl_2$ from a second syringe.

Data analysis

Most of data were fitted by non-linear regression with the program KALEIDAGRAPH (Abelbeck software) by using the appropriate equations, described in the Results section. The rates of processive polymerization were determined by kinetic simulation with the computer program KINSIM [28,29] and adjusting the kinetic parameters manually.

Trypsin digestion of Pol β–DNA complexes

After 1.7 μM Pol β had been complexed with 17 μM 25A/45TA and 3.4 μM 25A-19/45TA respectively in a solution containing 50 mM Tris/HCl, pH 8.0, 50 mM KCl, 10 % (v/v) glycerol, 1 mM DTT and 2.5 mM $MgCl_2$, 0.34 μM trypsin was added to each mixture (total volume 50 μl). Then 5 μl of reaction mixture was removed at 0, 5, 10, 20 and 30 min and quenched by mixing with a solution containing 50 mM Tris/HCl/2 % (w/v) SDS/200 mM DTT/0.1 % Bromophenol Blue/10 % (v/v) glycerol and heating the sample at 100 °C for 5 min. The samples were subjected to SDS/PAGE [12 % (w/v) gel] developed by the silver staining method [23].

RESULTS AND DISCUSSION

Kinetics of single-nucleotide gap-filling DNA synthesis

Kinetic analysis of single-nucleotide gap-filling DNA synthesis was first performed under conditions of excess DNA (25A-19/45TG, 250 nM) over the enzyme (80 nM by absorbance measurement) in the presence of saturating dCTP (50 μM). The resulting time course of the reaction showed a burst of dCTP incorporation followed by a linear steady-state phase similar to the curve obtained for duplex DNA substrate [20] (Figure 2). The pre-steady-state burst and overall biphasic shape of the curve observed in this experiment suggest that the rate-limiting step of single-nucleotide gap-filling synthesis is the dissociation of Pol β·nicked DNA after the incorporation of correct nucleotide.

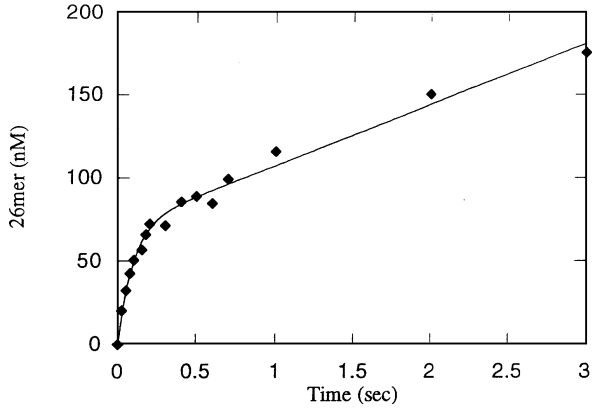

Figure 2 Burst kinetics of the incorporation of dCTP into 25A-19/45TG

Pol β (80 nM by absorbance measurement) and 25A-19/45TG (250 nM) were preincubated and rapidly mixed with 50 μM dCTP to begin the reaction. The reaction was quenched with 0.3 M EDTA (all final concentrations). The reaction conditions and methods of product analysis were as described in the Materials and methods section.

Effects of single-nucleotide gapped DNA on k_{pol}, K_d and catalytic efficiency

The pre-steady-state turnover number of Pol β, defined as k_{pol}, can be obtained by determining k_{obs} at various concentrations of dNTP under single turnover conditions (a 5-fold excess of enzyme over DNA was used in this study). The data were fitted to a single-exponential equation [eqn. (2)] to obtain k_{obs} at each concentration of dNTP, as shown in Figure 3(A):

$$[26\text{-mer}] = A[1-\exp(-k_{obs}\,t)] \quad (2)$$

where A is the initial concentration of the DNA substrate. The observed rates, k_{obs}, were plotted against dNTP concentrations, and the results were fitted with the hyperbolic equation (3) to determine k_{pol} and K_d, as shown in Figure 3(B):

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

where the apparent equilibrium dissociation constant, K_d, is the concentration of dNTP that gives the half-maximal rate of catalysis. The values of k_{pol} and K_d for incorporation of dCTP into 24A-19/45TG were determined as 12.5 ± 0.5 (S.D.) s^{-1} and 1.9 ± 0.2 μM respectively.

The values of k_{pol} and K_d were determined by the procedures described above for all four possible correct base pairs and all twelve possible incorrect base pairs. The single-nucleotide gapped DNA substrates used were 25A-19/45TA, 25A-19/45TT, 25A-19/45TG and 25A-19/45TC for the template bases A, T, G and C respectively. The results, along with the ratio k_{pol}/K_d and the fidelity (addressed below), are summarized in Table 1.

Complete kinetic analyses of all possible correct and incorrect base pairs have also been performed recently for non-gapped duplex DNA substrates [18]. Comparison of the results obtained with these two DNA substrates indicates that the use of single-nucleotide gapped DNA leads to a considerable decrease (by a factor of 6–29) in the K_d for correct dNTP. An example of such a comparison (between non-gapped 25A/45TA and gapped 25A-19/45TA) is shown in Table 2. As a consequence, the catalytic efficiency k_{pol}/K_d increases by 6–40-fold (for single-nucleotide gapped DNA relative to non-gapped DNA) for all four correct base pairs, as illustrated in Figure 4(A). Higher catalytic efficiency with single-nucleotide gapped DNA substrates was also observed for incorrect nucleotide incorporation, as shown in Figure 4(B). Interestingly, the difference here arose mainly from the difference in the value of k_{pol} (increases 2–200-fold with gapped DNA) rather than K_d.

The relatively high catalytic efficiency for the G:C base pair that was observed with normal duplex DNA substrate [18,19] was diminished with the gapped DNA substrates. Although the G:C still had the lowest K_d and the highest k_{pol}/K_d values, the differences became smaller and almost negligible with gapped DNA substrates. However, there is still a possibility that the values might have changed in the presence of other accessory proteins.

Effects of single-nucleotide gapped DNA on fidelity of Pol β

The contribution of k_{pol} and K_d to fidelity is different for single-nucleotide gap-filling and primer/template syntheses. With primer/template DNA substrates, Pol β selects the correct nucleotide against incorrect nucleotides mainly through k_{pol} [18]. The value of $k_{pol,c}/k_{pol,i}$ varied from 2800 to 85, whereas $K_{d,i}/K_{d,c}$

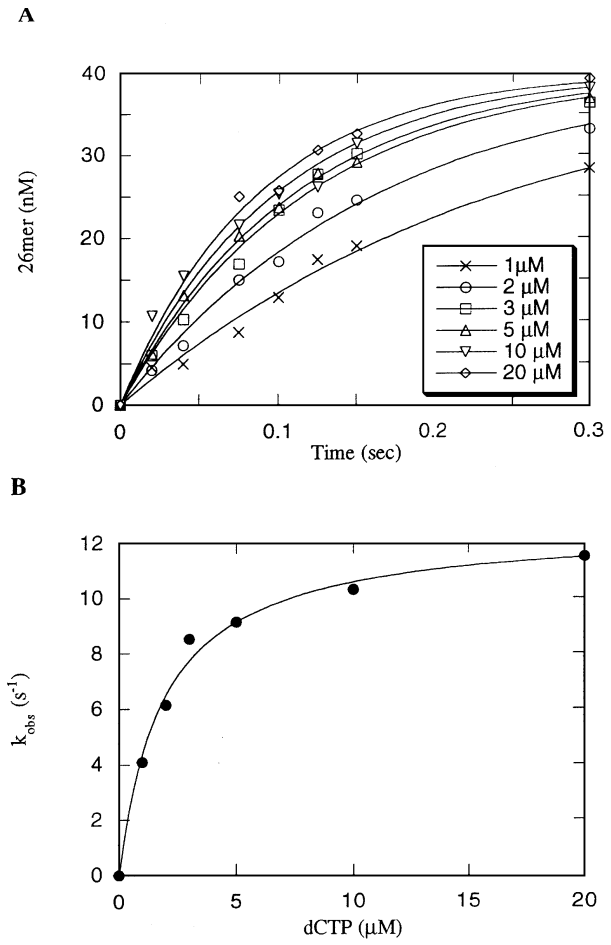

Figure 3 Pre-steady-state kinetics of dCTP incorporation into 25A-19/45TG catalysed by Pol β

(**A**) The product–time plot. DNA (40 nM) was incubated with 200 nM Pol β (by absorbance measurement) and rapidly mixed with 1, 2, 3, 5, 10 or 20 μM dCTP. The reaction was quenched with 0.3 M EDTA (final concentration) after 0.02, 0.04, 0.075, 0.1, 0.125, 0.15 or 0.3 s. The results were fitted to a single-exponential equation [eqn. (2)] to obtain k_{obs} for each dCTP concentration. (**B**) The dCTP concentration dependence of k_{obs}. k_{pol} and K_d were determined as 12.5 ± 0.5 s^{-1} and 1.9 ± 0.2 μM respectively by fitting to the hyperbolic equation [eqn. (3)].

Table 1 Kinetic parameters (means ± S.D.) for formation of all possible correct and incorrect base pairs and fidelity in single-nucleotide gap-filling DNA synthesis catalysed by Pol β

The DNA substrates used as base pair templates were 25A-19/45TX, shown in Figure 1(B). Fidelity is defined as $[(k_{pol}/K_d)_c + (k_{pol}/K_d)_i]/(k_{pol}/K_d)_i$, where subscripts c and i represent correct and incorrect nucleotide incorporation, respectively.

Base pair template : dNTP	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($M^{-} \cdot s^{-1}$)	Fidelity
A:T	21.9 ± 0.5	5.4 ± 0.7	4100000	
T:A	36.3 ± 4.4	8.5 ± 2.4	4300000	
G:C	12.5 ± 0.5	1.9 ± 0.2	6600000	
C:G	18.4 ± 0.8	3.6 ± 0.8	5100000	
A:C	0.23 ± 0.02	190 ± 50	1200	3400
A:G	0.16 ± 0.05	1600 ± 300	100	41000
A:A	0.031 ± 0.01	310 ± 50	100	41000
T:G	0.96 ± 0.01	620 ± 150	1500	2800
T:C	1.3 ± 0.12	510 ± 100	2500	1700
T:T	0.15 ± 0.01	1300 ± 200	120	37000
G:T	0.37 ± 0.09	650 ± 300	570	12000
G:A	0.019 ± 0.001	270 ± 40	70	93000
G:G	0.040 ± 0.002	360 ± 40	110	59000
C:A	0.24 ± 0.01	470 ± 70	510	10000
C:T	0.22 ± 0.01	877 ± 70	250	20000
C:C	0.026 ± 0.002	370 ± 50	70	73000

Table 2 Comparison of kinetic parameters of polymerization by Pol β and T7 Pol (exo$^-$) for various DNA substrates

Results are means ± S.D.

	Pol β		T7 pol (exo$^-$)	
DNA, substrate → product	k_{pol} (s^{-1})	K_d (μM)	k_{pol} (s^{-1})	K_d (μM)
25A/45TA → 26AT/45TA	16.7 ± 0.7	41 ± 6	330 ± 58	23 ± 5
25A-19/45TA → 26AT-19/45TA	21.9 ± 0.5	5.4 ± 0.7	240 ± 67	23 ± 3
25A-18/45TA → 26AT-18/45TA	16.9 ± 0.7	25.3 ± 4.6	–	–

varied over a substantially smaller range (44–5-fold). In contrast, the discrimination between incorrect and correct dNTPs is achieved through comparable contributions of $k_{pol,c}/k_{pol,i}$ and $K_{d,i}/K_{d,c}$ in single-nucleotide gap-filling synthesis. The value of $k_{pol,c}/k_{pol,i}$ varies from 708 to 28 and the value of $K_{d,i}/K_{d,c}$ varies from 340 to 35 (Table 1). Compared with the kinetic parameters with primer/template duplex DNA substrates, the K_d of correct nucleotide incorporation decreases and the k_{pol} of incorrect nucleotide incorporation increases with single-nucleotide gapped DNA substrates.

Our previous work with normal primer/template DNA duplex substrates demonstrated that the fidelity of Pol β in DNA replication is the lowest among DNA polymerases ever studied and is comparable to the fidelity of HIV reverse transcriptase [20,30]. With the use of gapped DNA substrates, it was hoped that the possible additional binding interactions between the N-terminal 8 kDa domain and the downstream primer could lead to higher fidelity in single-nucleotide gap filling. The observations of the higher k_{pol}/K_d ratio (mainly due to decreased K_d) for the correct dNTP in single-nucleotide gap-filling synthesis (relative to non-gapped DNA replication), as illustrated in Figure 4(A), suggested that the fidelity could improve with single-nucleotide gapped DNA. However, as shown in Figure 4(B), the ratios k_{pol}/K_d are also higher for incorrect dNTP incorporation into single-nucleotide gapped DNA substrates. As a consequence, the fidelity of single-nucleotide gap-filling synthesis remains essentially the same relative to that of DNA replication, as illustrated in Figure 5. The only exceptions are G:A and C:C mispairs, which showed 7- and 12-fold increases respectively in fidelity. In essence, the decrease in K_d of correct dNTP incorporation was masked by the increase in k_{pol} of incorrect dNTP incorporation in the single-nucleotide gap-filling synthesis.

There are two possible explanations for the relatively low fidelity of Pol β in the single-nucleotide gap-filling synthesis compared with the fidelity of other DNA polymerases in DNA replication. (1) Because it has been shown that Pol β belongs to the ancient nucleotidyltransferase superfamily [31,32]. Pol β might have started as a single nucleotidyl transfer enzyme and evolved into a DNA polymerase. However, in the base excision repair mode, it needed only to incorporate a single nucleotide. Thus the evolutionary pressure to synthesize Pol β with high fidelity might have been relatively low. (2) It is possible that the fidelity of Pol β is enhanced by accessory proteins *in vivo*.

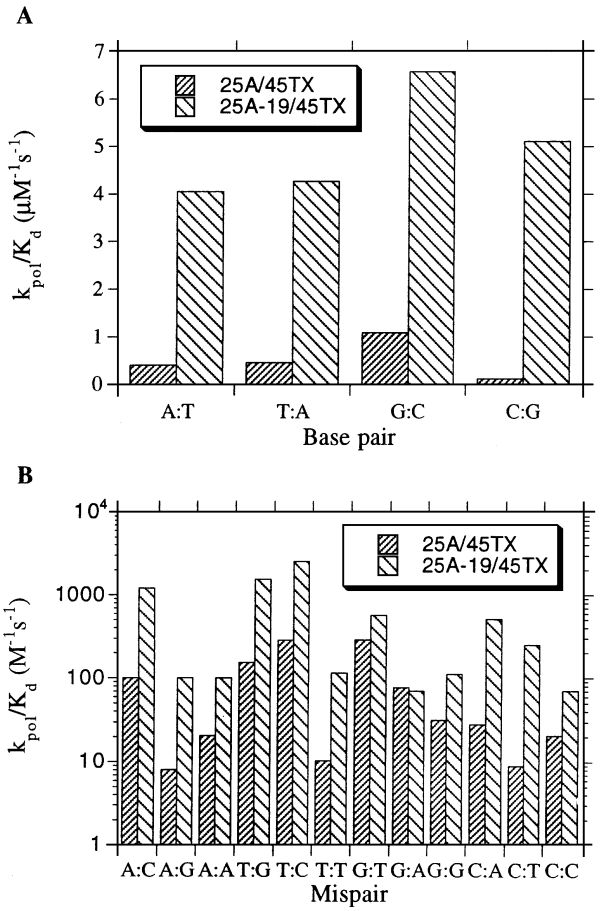

Figure 4 Comparison of substrate specificity (k_{pol}/K_d) for incorporation of four correct dNTPs (A) and formation of twelve possible mispairs (B) in single-nucleotide gap-filling synthesis and DNA replication

The results were taken from Table 1 and [18]. The base pair is represented as template:dNTP. In 25A/45X and 25A-19/45TX, X represents any template base: A, T, G or C.

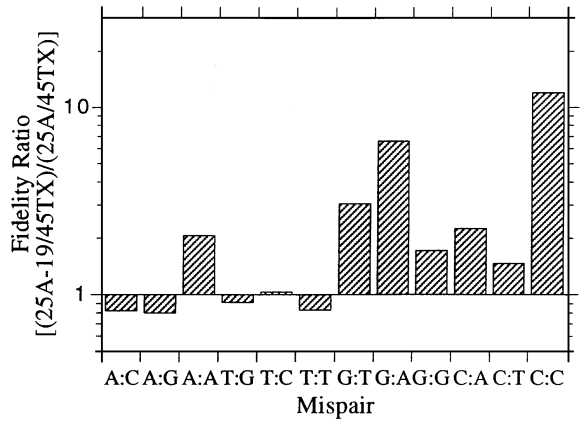

Figure 5 Ratio of fidelity of single-nucleotide gap-filling synthesis compared with DNA replication for Pol β

The mispair is represented as template:dNTP. The fidelity data were taken from Table 1 and [18]. In 25A/45X and 25A-19/45TX, X represents any template base: A, T, G or C.

Improvement in catalytic rate, fidelity and DNA-binding affinity has been observed for T7 [33] and T4 DNA polymerases [34–36] in the presence of accessory proteins. A possible candidate for a Pol β accessory protein is XRCC1, which is part of the machinery of the base excision repair system and has been shown to interact physically with Pol β [37]. It has also been found that Pol β forms a multiprotein base excision repair complex with DNA ligase I [38]. Further investigation is required to pursue these possibilities.

Control experiments with a different DNA polymerase and with two-nucleotide gapped DNA

To ascertain that the lower K_d of dNTP with single-nucleotide gapped DNA is a unique and inherent property of Pol β as a DNA repair polymerase involved in single-nucleotide gap-filling synthesis, the kinetic property of a different DNA polymerase, Sequenase 2.0 [a site-specific mutant of T7 DNA polymerase with diminished $3' \rightarrow 5'$ exonuclease activity, T7 Pol (exo$^-$)], was investigated with the same DNA substrates. As shown in Table 2, the enhanced binding of dNTP with single-nucleotide gapped DNA was not observed for T7 Pol (exo$^-$).

Another control experiment was performed with a two-nucleotide gapped DNA substrate, 25A-18/45TA, to determine whether the effect of a decreased K_d of dNTP for Pol β would disappear when the gap size was larger than one base. As shown by the results in Table 2, the K_d of dNTP for 25A-18/45TA was close to that for the non-gapped duplex DNA substrate. Similar results were obtained with DNA with even larger gap sizes (results not shown). These results indicate that only single-nucleotide gapped DNA seems to enhance the binding of dNTP.

Determination of the k_{off} of DNA with various sequence contexts and gap sizes

The processivity of DNA polymerases can be measured under two different conditions: (1) from the ratio k_{pol}/k_{off} obtained under single nucleotide incorporation [39]; (2) from the ratio k_{pol}/k_{off} obtained for multiple nucleotide incorporation [40]. To estimate the processivity of Pol β under first condition, we determined the k_{off} of DNA under various conditions and different sequence contexts. Because the kinetics of single nucleotide incorporation showed a burst of product formation followed by slow steady-state turnover (see Figure 2), the rate constant for DNA dissociation, k_{off}, can be approximated by the rate constant of the steady-state incorporation, k_{ss} [20,26,40,41]. The results obtained under steady-state conditions (approx. 850-fold excess of DNA over the enzyme used in this study) are reported in Table 3. Values of 1.0–2.0 s^{-1} were obtained for the four 26AN/45TX duplex DNA products (X and N represent dXMP template nucleotide and the newly incorporated dNMP respectively). The k_{off} values for the nicked DNA 26AN-19/45TX, which are products of single-base gapped DNA, fall in a similar but slightly smaller range (0.4–2.2 s^{-1}). The k_{off} decreased only insignificantly for three other DNA substrates with larger gaps, 26AT-18/45TA (1.0 s^{-1}), 26AT-21/50TA (0.9 s^{-1}), and 26AT-18/50TA (0.6 s^{-1}). Overall, under conditions of single nucleotide incorporation, k_{off} shows a smaller dependence (less than 5-fold) on the sequence of DNA at the site of incorporation, and still smaller dependence on the type of DNA substrates (gapped DNA versus non-gapped duplex DNA, or gapped DNA with different gap sizes).

As an independent confirmation, the k_{off} value of the 26AA/45TT duplex has been determined by DNA competition/trapping experiment with a rapid-quench apparatus. The result obtained (2.2 s^{-1}) agrees with that obtained from the steady-state measurement (2.0 s^{-1}).

Table 3 Dissociation rates of DNA

The dissociation rates of DNA were determined for the product under steady-state conditions as described in the Materials and methods section, with the following modification. The reaction solution contained 0.3 nM Pol β, 250 nM DNA substrates, 200 μM dNTP and 2.5 mM $MgCl_2$. Results are means ± S.D.

Substrate → product	Dissociation rate (s^{-1})
25A/45TA → 26AT/45TA	1.6 ± 0.4
25A/45TT → 26AA/45TT	2.0 ± 0.2*
25A/45TG → 26AC/45TG	1.0 ± 0.3
25A/45TC → 26AG/45TC	1.4 ± 0.3
25A-19/45TA → 26AT-19/45TA	1.3 ± 0.2
25A-19/45TT → 26AA-19/45TT	2.2 ± 0.3
25A-19/45TG → 26AC-19/45TG	0.4 ± 0.1
25A-19/45TC → 26AG-19/45TC	1.2 ± 0.2
25A-18/45TA → 26AT-18/45TA	1.0 ± 0.3
25A-21/50TA → 26AT-21/50TA	0.9 ± 0.3
25A-18/50TA → 26AT-18/50TA	0.6 ± 0.2

* This is different from the k_{off} value reported previously [20]. The previous value, 0.3 s^{-1}, should be considered inaccurate because it was estimated from the steady-state phase of a burst experiment in which the DNA was in only a small excess over the enzyme.

Table 4 Comparison of processivity (k_{pol}/k_{off}) determined under two different conditions for DNA replication and gap-filling synthesis

Results are means ± S.D.

Substrate → product	k_{pol} (s^{-1})	k_{off} (s^{-1})	k_{pol}/k_{off}
Single nucleotide incorporation			
25A/45TA → 26AT/45TA	16.7*	1.6‡	10
25A/45TT → 26AA/45TT	24.1*	2.0‡	12
25A/45TG → 26AC/45TG	9.4*	1.0‡	9.4
25A/45TC → 26AG/45TC	13.5*	1.4‡	10
25A-19/45TA → 26AT-19/45TA	21.9†	1.3‡	17
25A-19/45TT → 26AA-19/45TT	36.3†	2.2‡	17
25A-19/45TG → 26AC-19/45TG	12.5†	0.4‡	31
25A-19/45TC → 26AG-19/45TC	18.4†	1.2‡	15
25A-18/45TA → 26AT-18/45TA	17	1.0‡	17
Multiple nucleotide incorporation			
25A/45TA →	18	10	1.8
25A-18/45TA →	17	0.3	57

* From [18].
† From Table 1.
‡From Table 3.

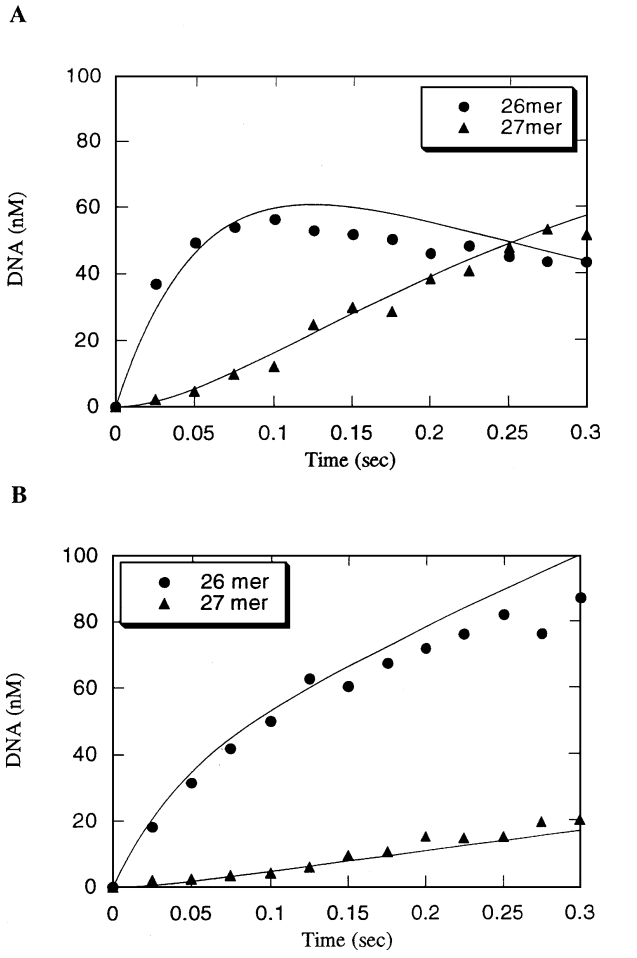

Figure 6 Processive polymerization by Pol β on 25A-18/45TA (A) and 25A/45TA (B)

(**A**) Pol β (88 nM) (active site) and 150 nM DNA were incubated before being mixed rapidly with 250 μM dTTP and 20 μM dCTP. The reaction was quenched with 0.3 M EDTA (final concentration) after the indicated times. The results represent the formation of 26-mer and 27-mer. The solid lines represent best fits obtained from computer simulation with a mechanism consisting of a series of two nucleotide incorporation at rates of 17 and 4 s^{-1} respectively; DNA dissociation rates of 0.3, 0.3 and 0.6 s^{-1} for 25-18/45, 26-18/45 and 27-18/45 respectively; and DNA association rates of 1.5×10^8, 7.5×10^7 and 5×10^7 $M^{-1} \cdot s^{-1}$ respectively. (**B**) Pol β (80 nM) (active site) and 300 nM DNA were incubated before being mixed rapidly with 250 μM dTTP and 100 μM dCTP. The same simulation gave two nucleotide incorporation rates of 18 and 2 s^{-1} respectively; DNA dissociation rates of 3.5, 10 and 10 s^{-1} for 25/45, 26/45 and 27/45 respectively; and DNA association rates of 5×10^7, 5×10^7 and 5×10^7 $M^{-1} \cdot s^{-1}$ respectively.

Whereas Pol β dissociates at comparable rates from different types of DNA, T7 Pol (exo$^-$) dissociates at a significantly faster rate from the nicked DNA 26AT-19/45TA (28 s^{-1}) than single-nucleotide gapped DNA 26AT-18/45TA (0.3 s^{-1}) or normal duplex DNA 26AT/45TA (0.25 s^{-1}). Because T7 Pol (exo$^-$) has a high k_{pol}/k_{off} ratio and displays a clear distinction between the burst and the steady-state phases, the rates of DNA dissociation for T7 Pol (exo$^-$) were directly estimated from the rates of the linear phase after the burst under single nucleotide incorporation conditions. The enhanced dissociation of T7 Pol (exo$^-$) from nicked DNA is probably due to the nicked DNA's being the final product of DNA synthesis. The lack of such an enhancement for Pol β could be due to the fact that nicked DNA mimics the substrate for the auxiliary deoxyribose phosphatase activity of Pol β [17]. The apparent conformational similarity of the recently published structures for single-base gapped DNA substrate–Pol β and nicked DNA–Pol β complexes also indicates that the enzyme might have similar affinities towards both DNA types [42].

Comparison of processivity between duplex and gapped DNA substrates

The processivity under the single nucleotide incorporation condition can be readily calculated for both duplex DNA substrates and two-nucleotide gapped DNA substrates from the k_{pol} and the k_{off} data obtained above and in Ahn et al. [18], as listed in Table 4. The meaning of the ratio k_{pol}/k_{off} is only theoretical, because the enzyme is forced to dissociate from DNA after a single catalytic cycle and does not actually 'polymerize'. We

Table 5 Kinetic parameters for incorporation of nucleoside 5′-[α-thio]triphosphates into DNA substrates catalysed by Pol β

The thio effects listed here might be overestimated owing to the use of the mixture of isomers, as explained in [21]. Results are means ± S.D.

	25A/45TT			25A-19/45TT		
Base pair template:dNTP	$k_{pol,thio}$ (s^{-1})	$K_{d,thio}$ (μM)	$k_{pol}/k_{pol,thio}$	$k_{pol,thio}$ (s^{-1})	$K_{d,thio}$ (μM)	$k_{pol}/k_{pol,thio}$
T:A	5.1 ± 0.1	40 ± 6	4.8*	5.6 ± 0.3	7.0 ± 1.2	6.6
T:G	0.011 ± 0.001	960 ± 50	12*	0.17 ± 0.01	490 ± 50	5.6

* These values are slightly different from those reported by Werneburg et al. [20] owing to slightly different experimental conditions.

have previously reported that Pol β is weakly processive ($k_{pol}/k_{off} = 30$) for duplex DNA substrates [20]. With the more accurate k_{off} values determined in the present work, the k_{pol}/k_{off} ratios are somewhat lower and fall in the range 9–12 for non-gapped DNA substrates. The processivity for single-nucleotide gapped DNA substrates is only marginally higher ($k_{pol}/k_{off} = 15$–31).

We have also determined the processivity with multiple nucleotide incorporation (processive polymerization) experiments, which are more meaningful. In these experiments successive incorporation of dTTP and dCTP into 25A-18/45TA and 25A/45TA were permitted. The concentration of DNA was in 2–4-fold excess over the enzyme concentration and a short time course of polymerization was allowed to ensure that the observed kinetics of polymerization satisfy a single enzyme-binding event [41]. The enzyme should bind to and dissociate from only one DNA substrate molecule; binding to other DNA molecules during the time course of processive polymerization should be limited. Kinetic simulation with a reaction mechanism consisting of a series of dNTP incorporation, DNA dissociation and association was used to fit the accumulation and disappearance of products [40], as shown in Figure 6. The results, presented in the last two rows of Table 4, indicate that the processivity of Pol β in gap-filling synthesis (57) is approx. 30-fold that of the corresponding value for DNA replication with duplex DNA (1.8) under processive polymerization conditions. These results are in good agreement with results of the steady-state experiments reported previously [43,44]. The enhanced processivity for gapped DNA substrates is caused by a higher affinity (lower k_{off}) for gapped DNA than for duplex DNA, whereas the rates of polymerization (k_{pol}) are essentially the same. The reason for the higher k_{off} for duplex DNA might be similar to that proposed for HIV reverse transcriptase [40]. Both the higher affinity towards gapped DNA substrate and the low processivity with duplex DNA support the biological function of Pol β in DNA repair.

Observation of a rate-limiting conformational change step in single-nucleotide gap-filling synthesis

The thio effect, defined as the ratio of $k_{pol}/k_{pol,thio}$, can be used to probe whether the rate-limiting step in the single-nucleotide gap-filling synthesis is changed compared with that with non-gapped DNA substrates. The observed thio effects for the incorporation of correct and incorrect dNTP into a non-gapped duplex DNA (25A/45TT) and a single-nucleotide gapped DNA (25A-19/45TT) are listed in Table 5. The thio effect for single-nucleotide gap-filling synthesis (6.6) is similar to the value for DNA replication (4.8). The thio effect for the incorporation of incorrect dGTP into 25A-19/45TT (5.6) is one-half of that for 25A/45TT (12). The small changes in thio effect suggest that the rate-

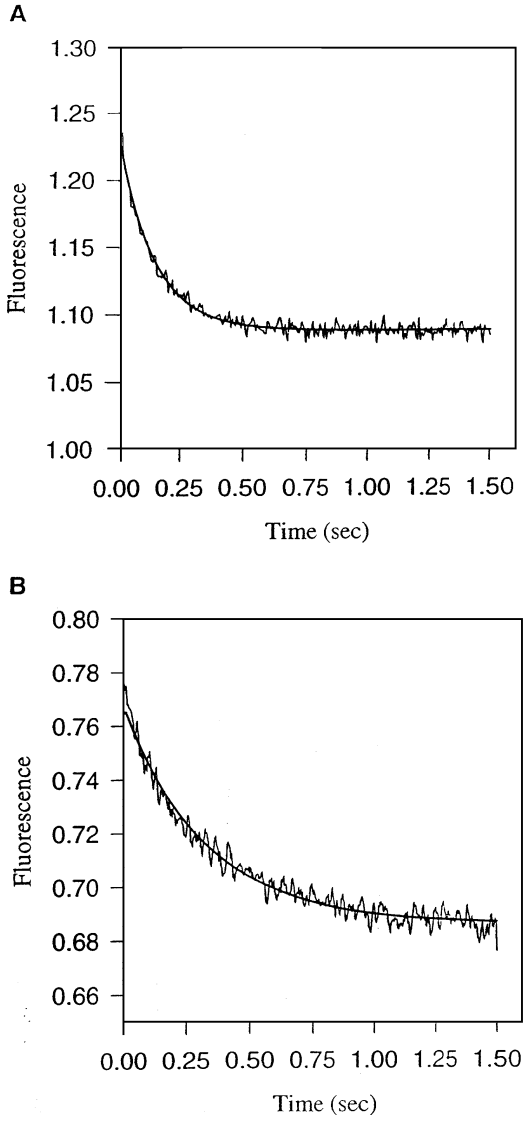

Figure 7 Stopped-flow fluorescence assay

(**A**) Incorporation of dTTP opposite template 2-aminopurine with single-base gapped DNA substrate. Experiment was performed as described in the Materials and methods section. The data were fitted to a single exponential to yield the observed rate constant of 7.0 ± 0.2 s^{-1}. (**B**) Addition of dTTP opposite template 2-aminopurine with single-base gapped DNA substrate incorporated with ddAMP at the 3′ end of the primer. The data were fitted to a single exponential which gave the observed rate constant of 3.0 ± 0.1 s^{-1}.

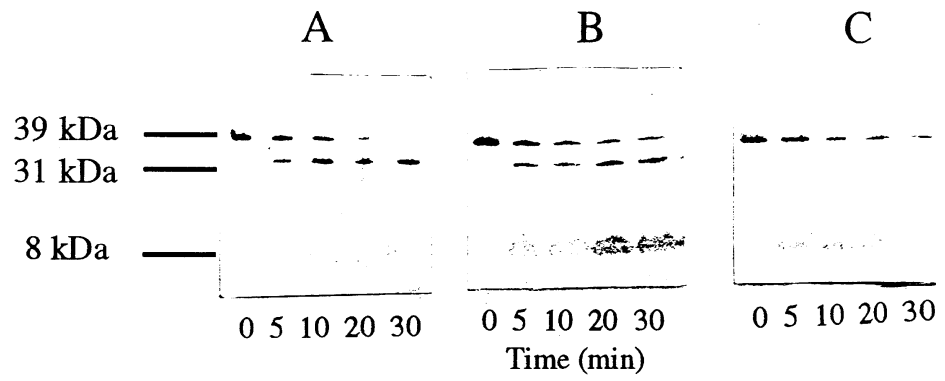

Figure 8 Trypsin digestion of Pol β and Pol β/DNA complexes

Pol β (**A**), Pol β and 25A/45TA (**B**) and Pol β and 25A-19/45TA (**C**). Trypsin was added to the solution, and the proteolysis was terminated at 0, 5, 10, 20 or 30 min as described in the Materials and methods section. The resulting solutions were subjected to SDS/PAGE analysis.

limiting step should not have been altered by the use of single-nucleotide gapped DNA substrates.

However, thio effect is only a qualitative and indirect method to assess the rate-limiting step. We have reported the direct observation of a fast and a rate-limiting conformational change by stopped-flow fluorescence with non-gapped duplex DNA [21]. We have now performed such experiments with single-nucleotide gapped DNA. As shown in Figure 7(A), the changes in fluorescence with single-gapped DNA substrate can be fitted to a single exponential. No significant improvement is achieved when fitted to a double exponential. The rate of this phase (7.0 s^{-1}) corresponds to product formation as confirmed by a fast chemical quench experiment (8.2 s^{-1}). Thus this phase corresponds to the slow phase observed with non-gapped duplex DNA, with the exception that the fluorescence amplitude decreases with time. This slow phase was also observed with dideoxynucleotide-terminated primer (Figure 7B), although the observed rate without a 3′ hydroxy group in the dideoxynucleotide-terminated primer is approx. one-half (3.0 s^{-1}). This indicates that the rate-limiting step for single gap-filling synthesis is also a conformational change step. The fast phase observed with the regular duplex DNA substrate was not clearly detected with the gapped DNA substrate.

Conformational differences between Pol β complexes with gapped DNA and duplex DNA substrates

The absence of the fast conformational change step with gapped DNA substrate suggests that Pol β possesses different conformations initially when it binds to the gapped-DNA substrate and the normal DNA substrate. On the basis of the comparison between the structures of Pol β complexed with duplex DNA and ddCTP [45] and Pol β complexed with gapped DNA and ddCTP published recently [42], the 8 kDa N-terminal domain seems to be responsible for the difference. In the duplex DNA complex this domain is flexible [45], whereas in the gapped DNA complex this domain exists in a closed conformation [42]. Thus the fast conformational change observed with the normal non-gapped duplex DNA, but not with gapped substrate, might be caused by the closure of the 8 kDa domain. The proposed closed conformation of Pol β on binding to gapped DNA is further supported by the trypsin digestion of Pol β complexed with normal duplex and with gapped DNA, as shown in Figure 8. In this experiment Pol β was incubated with a 10-fold excess of 25A/45TA (Figure 8B) or with a 2-fold excess of 25A-19/45TA. Under these conditions 80 % of Pol β should exist in the bound form. The results show that when Pol β is complexed with single-base gapped DNA, the rate of its degradation into the 31 and 8 kDa domains by trypsin is lower than when it is complexed with normal duplex DNA.

In conclusion, Pol β displays different kinetic and structural properties towards the gapped DNA substrates from those toward non-gapped DNA substrates. The results support the suggested role of Pol β in base excision repair.

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REFERENCES

1 Lindahl, T. (1993) Nature (London) **362**, 709–715
2 Friedberg, E. C., Walker, G. C. and Siede, W. (eds.) (1995) DNA Repair and Mutagenesis, pp. 407–593, ASM Press, New York
3 Lehmann, A. R. (1995) Trends Biochem. Sci. **20**, 402–405
4 Shinohara, A. and Ogawa, T. (1995) Trends Biochem. Sci. **20**, 381–391
5 Fishel, R., Lescoe, M. K., Rao, M. R. S., Copeland, N. G., Jenkins, N. A., Garber, J., Kane, M. and Kolodner, R. (1993) Cell **75**, 1027–1038
6 Leach, F. S., Nicolaides, N. C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomäki, P., Sistonen, P., Aaltonen, L. A., Nyström-Lahti, M. et al. (1993) Cell **75**, 1215–1225
7 Modrich, P. (1994) Science **266**, 1959–1960
8 Parsons, R., Li, G.-M., Longley, M. J., Fang, W., Papadopoulos, N., Jen, J., de la Chapelle, A., Kinzler, K. W., Vogelstein, B. and Modrich, P. (1993) Cell **75**, 1227–1236
9 Parsons, R., Li, G.-M., Longley, M. J., Modrich, P., Liu, B., Berk, T., Hamilton, S. R., Kinzler, K. W. and Vogelstein, B. (1995) Science **268**, 738–740
10 Tanabe, K., Bohn, E. W. and Wilson, S. H. (1979) Biochemistry **18**, 3401–3406
11 Dianov, G., Price, A. and Lindahl, T. (1992) Mol. Cell. Biol. **12**, 1605–1612
12 Singhal, R. K., Prasad, R. and Wilson, S. H. (1995) J. Biol. Chem. **270**, 949–957
13 Wiebauer, K. and Jiricny, J. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5842–5845
14 Narayan, S., He, F. and Wilson, S. H. (1996) J. Biol. Chem. **271**, 18508–18513
15 Srivastava, D. K., Rawson, T. Y., Showalter, S. D. and Wilson, S. H. (1995) J. Biol. Chem. **270**, 16402–16408
16 Sobol, R. W., Horton, J. K., Kühn, R., Gu, H., Singhal, R. K., Prasad, R., Rajewsky, K. and Wilson, S. H. (1996) Nature (London) **379**, 183–186
17 Matsumoto, Y. and Kim, K. (1995) Science **269**, 699–702
18 Ahn, J., Werneburg, B. G. and Tsai, M.-D. (1997) Biochemistry **36**, 1100–1107
19 Kraynov, V., Werneburg, B. G., Zhong, X., Lee, H., Ahn, J. and Tsai, M.-D. (1997) Biochem. J. **323**, 103–111
20 Werneburg, B. G., Ahn, J., Zhong, X., Hondal, R. J., Kraynov, V. and Tsai, M.-D. (1996) Biochemistry **35**, 7041–7050
21 Zhong, X., Patel, S. S., Werneburg, B. G. and Tsai, M.-D. (1997) Biochemistry **36**, 11891–11900

22 Date, T., Yamaguchi, M., Hirose, F., Nishimoto, Y., Tanihara, K. and Matsukage, A. (1988) Biochemistry **27**, 2983–2990
23 Poehling, H. M. and Neuhoff, V. (1981) Electrophoresis **2**, 141–147
24 Casas-Finet, J. R., Kumar, A., Morris, G., Wilson, S. H. and Karpel, L. (1991) J. Biol. Chem. **266**, 19618–19625
25 Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G. and Rutter, W. J. (1970) Science **170**, 447–448
26 Kuchta, R. D., Mizrahi, V., Benkovic, P. A., Johnson, K. A. and Benkovic, S. J. (1987) Biochemistry **26**, 8410–8417
27 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY
28 Barshop, B. A., Wrenn, R. F. and Frieden, C. (1983) Anal. Biochem. **130**, 134–145
29 Zimmerle, C. T. and Frieden, C. (1989) Biochem. J. **258**, 381–387
30 Kunkel, T. A. and Alexander, P. S. (1986) J. Biol. Chem. **261**, 160–166
31 Holm, L. and Sander, C. (1995) Trends Biochem. Sci. **20**, 345–347
32 Matsukage, A., Nishikawa, K., Tatsuo, O., Seto, Y. and Yamaguchi, M. (1987) J. Biol. Chem. **262**, 8960–8962
33 Tabor, S., Huber, H. E. and Richardson, C. C. (1987) J. Biol. Chem. **262**, 16212–16223
34 Muun, M. M. and Alberts, B. M. (1991) J. Biol. Chem. **266**, 20024–20033
35 Muun, M. M. and Alberts, B. M. (1991) J. Biol. Chem. **266**, 20034–20044
36 Young, M. C., Reddy, M. K. and von Hippel, P. H. (1992) Biochemistry **31**, 8675–8690
37 Caldecott, K. W., Aoufouchi, S., Johnson, P. and Shall, S. (1996) Nucleic Acids Res. **24**, 4387–4394
38 Prasad, R., Singhal, R. K., Srivastava, D. K., Molina, J. T., Tomkinson, A. E. and Wilson, S. H. (1996) J. Biol. Chem. **271**, 16000–16007
39 Johnson, K. A. (1993) Annu. Rev. Biochem. **63**, 685–713
40 Kati, W. M., Johnson, K. A., Jerva, L. F. and Anderson, K. S. (1992) J. Biol. Chem. **267**, 25988–25997
41 Patel, S. S., Wong, I. and Johnson, K. A. (1991) Biochemistry **30**, 511–525
42 Sawaya, M. R., Prasad, R., Wilson, S. H., Kraut, J. and Pelletier, H. (1997) Biochemistry **36**, 11205–11215
43 Wang, T. S.-F. and Korn, D. (1982) Biochemistry **21**, 1597–1608
44 Singhal, R. K. and Wilson, S. H. (1993) J. Biol. Chem. **268**, 15906–15911
45 Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H. and Kraut, J. (1994) Science **264**, 1891–1903

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