Increased Activity and Fidelity of DNA Polymerase β on Single-nucleotide Gapped DNA*

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DNA polymerase β (pol β) is an error-prone polymerase that plays a central role in mammalian base excision repair. To better characterize the mechanisms governing rat pol β activity, we examined polymerization on synthetic primer-templates of different structure. Steady-state kinetic analyses revealed that the catalytic efficiency of pol β ($k_{cat}/K_{m, dNTP}$) is strongly influenced by gap size and the presence of a phosphate group at the 5'-margin of the gap. pol β exhibited the highest catalytic efficiency on 5'-phosphorylated 1-nucleotide gapped DNA. This efficiency was 5500 times higher than on non-phosphorylated 1-nucleotide and 6-nucleotide (with or without PO₄) gapped DNAs and 2500 times higher than on primer-template with no gaps. The nucleotide insertion fidelity of pol β, as judged by its ability to form G-N mispairs, was also higher (10–100 times) on 5'-phosphorylated single-nucleotide gapped DNA compared with the other DNA substrates studied. These data suggest that a primary function of mammalian pol β is to fill 5'-phosphorylated 1-nucleotide gaps.

DNA polymerase β (pol β)† plays a central role in mammalian base excision repair (BER (1–5)). pol β is a monomeric 36-kDa enzyme organized into a carboxyl-terminal 31-kDa domain that includes the polymerase active site and an amino-terminal 8-kDa domain that participates in DNA binding and harbors 5'-deoxyribose phosphodiesterase (lyase) activity (6, 7). The presence of both polymerase and lyase activities suggests that pol β catalyzes two steps in the "short-patch" BER pathway: removal of a 5'-deoxyribose phosphate intermediate and subsequent filling of the resultant 1 nt gap (4, 6). pol β has also been implicated in "long-patch" BER (4) and may function in meiosis (8) and nucleotide excision repair (9, 10).

The biochemical activities of purified pol β are consistent with a role in gap-filling DNA synthesis. Early studies showed that pol β is non-processive on single-stranded DNA templates, prefers short-gapped DNA substrates, and is capable of filling gaps to completion (11–16). More recently, Wilson and colleagues (17) observed that pol β fills short gaps (2–6 nt) by a processive mechanism that requires a PO₄ group at the 5'-margin of the gap. Binding of pol β to these short-gapped substrates is also strongly enhanced by the presence of a 5'-PO₄ (18). These experiments, together with recent structural data, suggest a model in which pol β binding to gapped DNA is mediated by interactions between the 8-kDa domain of pol β and the 5'-PO₄ at the downstream margin of the gap (7, 18). Processive DNA synthesis on short (2–6 nt) gaps is consistent with roles for pol β in long-patch BER (4) and in the completion of gap-filling synthesis initiated by other cellular DNA polymerases (9, 10, 14–16).

Although DNA polymerization by pol β on single-stranded and short-gapped DNAs is understood in some detail, much less is known about pol β activity on its short-patch BER substrate, 1-nt gapped DNA. The model of pol β binding through its 8-kDa domain to the 5'-PO₄ in short-gapped DNA does not appear to apply to 1-nt gaps; reducing the gap size from 5 to 1 nt decreases binding slightly, and the 5'-phosphorylation requirement is lost (18). This suggests that pol β may interact with 1-nt gapped DNA by a distinct mechanism. To better characterize the parameters governing pol β activity on 1-nt gapped DNA, we examined the steady-state kinetics of DNA polymerization on synthetic primer-templates of different structure. We show that the catalytic efficiency ($k_{cat}/K_{m, dNTP}$) and nucleotide insertion fidelity of pol β are strongly influenced by gap size and that the 5'-phosphorylation requirement is retained for these activities even on 1-nt gapped DNA. These data have important implications for models of pol β DNA binding and provide biochemical evidence that 5'-phosphorylated 1-nt gapped DNA is the preferred substrate for pol β.

EXPERIMENTAL PROCEDURES

Materials—Recombinant rat DNA polymerase β was purified as described previously (19). All oligonucleotides were synthesized and high-pressure liquid chromatography-purified by Operon Technologies. 5'-32P labeling of the primers was performed with γ-32P ATP (3000 Ci/mmol; Amersham Corp.) using T4 polynucleotide kinase (U. S. Biochemical Corp.) according to the manufacturer’s protocol. Labeled primers were separated from excess γ-32P ATP after labeling by gel filtration through 0.5-ml Sephadex G-50 (Pharmacia Biotech Inc., DNA grade) spin columns. 2'-Deoxyribonucleoside 5'-triphosphates (dNTPs) were from Calbiochem or Pharmacia. Concentrations of individual dNTPs were determined by UV spectroscopy (Beckman DU65). Protein concentrations were determined by the method of Bradford (Bio-Rad) according to the manufacturer’s protocol. All other reagents were of the highest grade available from Fisher Scientific or Sigma.

Primer-Template—Primer-templates of different structure were constructed from synthetic oligodeoxyribonucleotides (see Fig. 1). Hybridizations were done by mixing equimolar amounts of the required oligonucleotides in 250 mM KCl, 50 mM Tris-HCl, pH 8.0 (22 °C) and incubating sequentially at 65 °C (10 min), 37 °C (10 min), 22 °C (10 min), and 0 °C (10 min). Annealing efficiencies were >95%, as evidenced by mobility shifts on non-denaturing polyacrylamide gel elec-
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**RESULTS**

Design of Recessed and Gapped DNA Substrates—To examine pol β activity on DNA substrates of different structure, a series of primer-templates was constructed (Fig. 1). These DNAs all contained the same 46-mer template sequence based on a region of bacteriophage dX174 DNA used in previous fidelity studies (Refs. 20 and 23, and references therein). All of the substrates also contained the same 5′-32P-labeled primer hybridized to template residues 22–41. This places the primer 3′-OH terminus such that polymerization of the first dNTP

![](image)

**FIG. 3. Gel assay of nucleotide insertion kinetics by pol β.** The DNA substrates gap-1 (A) or P-gap-1 (B) were incubated with pol β in the presence of increasing concentrations of a single dNTP under steady-state conditions as described under “Experimental Procedures.” Products were resolved by urea-PAGE and then visualized and quantified using a PhosphorImager. Extensions of [5′-32P]-labeled primers to 21-mer products reflect the insertion of a single dNMP opposite template G21 in the DNA (Fig. 1). pol β concentrations and incubation times were adjusted to optimize detection of primer extension products; only data obtained from reactions conducted in the steady state (i.e., >70% primer extension) were used in the kinetic analyses in Tables I and II. A, dCTP reactions: 0.3 nM pol β, 3-min incubations; dGTP, dATP, and dTTP reactions: 6 nM pol β, 15-min incubations. B, dCTP reactions: 0.1 nM pol β, 3-min incubations; dGTP, dATP, and dTTP reactions: 2 nM pol β, 6-min incubations. In reactions containing the incorrect nucleotides (dGTP, dATP, or dTTP), product 21-mers were confirmed to result from true misinsertions (and not correct insertions of potential trace dCTP contaminants) by comparing electrophoretic mobilities with synthetic [5′-32P]-labeled markers of identical sequence containing 3′ terminal G, A, or T residues (lanes circled with an asterisk in B). Each 21-mer primer was 5′-phosphorylated at a characteristic rate, with the correct C-containing 21-mer running 1.5–3 mm ahead of the incorrect G-, A-, and T-containing 21-mers. The 22-mer products formed on gap-1 (A) presumably result from partial displacement of the downstream oligonucleotide and incorporation of the next correct nucleotide dATP (see Fig. 1).
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Table I

| DNA substrate a | $K_{m, dCTP}$ (µM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_{m, dCTP}$ (s⁻¹·µM⁻¹) |
|-----------------|--------------------|-----------------|----------------------------------|
| recessed        | 170 ± 40           | 0.6 ± 0.3       | 4 × 10³                           |
| gap-6           | 290 ± 130          | 0.3 ± 0.2       | 1 × 10³                           |
| P-gap-6         | 50 ± 20            | 1.0 ± 3.0       | 2 × 10⁴                           |
| gap-1           | 60 ± 10            | 1.2 ± 0.5       | 2 × 10⁴                           |
| P-gap-1         | 0.2 ± 0.1          | 2.0 ± 0.4       | 1 × 10⁷                           |

a Sequences and structures of the DNA substrates are shown in Fig. 1.
b Calculated using total pol β protein concentration.
c Values in parentheses indicate the number of independent experiments used for each analysis.

Effect of DNA Substrate Structure on pol β Catalytic Efficiency

Kinetic experiments were run, quantified, and analyzed as outlined under “Experimental Procedures.” Fig. 3 shows representative gels used for analysis.

Effect of DNA substrate structure on pol β fidelity

Experiments were run as described under “Experimental Procedures.” Fig. 3 shows representative gels used for analysis. Mispair formation frequencies were calculated from the initial slopes of Michaelis-Menten curves using the formula $f_{mis} = (k_{cat}/K_{m,dCTP})_{correct}/(k_{cat}/K_{m,dCTP})_{incorrect}$ (21, 22), where “correct” corresponds to extension in the presence of dCTP to form the G-C base pair (Table II).

Table II

| DNA substrate a | G-G      | G-A      | G-T      |
|-----------------|----------|----------|----------|
| recessed        | 1.0 (±0.5) × 10⁻⁴ | 1.6 (±0.1) × 10⁻³ | 1.0 (±0.3) × 10⁻⁵ |
| gap-6           | 4.2 (±4.0) × 10⁻⁵ | 2.0 (±1.5) × 10⁻³ | 9.0 (±4.0) × 10⁻⁴ |
| P-gap-6         | 5.0 (±4.0) × 10⁻⁵ | 1.3 (±0.5) × 10⁻³ | 1.8 (±1.0) × 10⁻⁵ |
| gap-1           | 8.0 (±4.0) × 10⁻⁵ | 2.0 (±1.0) × 10⁻³ | 6.0 (±1.5) × 10⁻³ |
| P-gap-1         | 2.0 (±2.0) × 10⁻⁶ | 6.0 (±3.0) × 10⁻⁵ | 1.0 (±0.2) × 10⁻⁵ |

a Sequences and structures of the DNA substrates are shown in Fig. 1.
b Values in parentheses indicate the number of independent experiments used for each analysis.

Values for pol β (40-170 µM; Table I and Ref. 21) are substantially higher than those observed for other DNA polymerases on similar or identical primer-templates in similar steady-state kinetic assays (typically 0.1-10 µM; Refs. 20–22, and references therein). The unusually high $K_{m,dCTP}$ values observed on the recessed and gap-6 primer-templates suggested that these DNAs were relatively poor substrates for pol β.

In a manner reminiscent of its effect on processivity (Fig. 2 and Ref. 17) and DNA binding (18), 5'-phosphorylation of gap-6 resulted in a modest reduction in $K_{m,dCTP}$ and concomitant increase in overall catalytic efficiency ($k_{cat}/K_{m,dCTP}$). A similar decrease in $K_{m,dCTP}$ and increase in catalytic efficiency occurred when the gap size was reduced from 6 to 1 nt in the absence of a 5'-PO₄ (Table I; compare gap-6 with gap-1). Most striking, however, was the dramatic effect of 5'-phosphorylation on the 1-nt gapped substrate, where addition of a 5'-PO₄ resulted in a 500-fold increase in catalytic efficiency (compare gap-1 to P-gap-1). Thus, the relative catalytic efficiencies of pol β on the different DNA substrates were P-gap-1 > gap-1 > gap-6 > recessed. pol β was some 10,000 and 2,500 times more efficient on P-gap-1 than on the gap-6 and recessed DNA substrates, respectively. As noted above, this increase in catalytic efficiency resulted primarily from a decrease in $K_{m,dCTP}$ although $k_{cat}$ values were also slightly higher on P-gap-1. The $k_{cat}$ value of 0.6 s⁻¹ observed on the recessed DNA substrate is very similar to the value of 0.3 s⁻¹ reported for a different pol β preparation on a different recessed primer-template (24).

Effect of DNA Substrate Structure on pol β Fidelity—The nucleotide insertion fidelity of pol β was determined on the same series of DNA substrates using a “standing start” (22) kinetic fidelity assay (Fig. 3 and Table II). The frequencies of nucleotide misinsertions opposite the template G²¹ residue were similar for all substrates except P-gap-1. The fidelity of pol β on P-gap-1 was 100, 50, and 30 times higher for G-T, G-G,
and G-A mispair formation, respectively, compared with the recessed substrate. G-T and G-A mispairs were formed ~10-fold more readily than G-G mispairs on all of the DNA substrates studied.

**DISCUSSION**

pol β plays a central role in mammalian short-patch BER (1–5). This suggests that a preferred substrate for pol β might be 5'-phosphorylated 1-nt gapped DNA. We examined the DNA substrate preferences of purified rat pol β in steady-state kinetic assays using synthetic DNAs of different structure. We show that pol β prefers 5'-phosphorylated 1-nt gapped DNA as substrate with relative catalytic efficiencies on P-gap-1 => gap-1 ~ P-gap-6 > gap-6 ~ recessed (Table I). The efficiency of pol β on P-gap-1 DNA was 500–10,000 times higher than on the other DNA substrates examined. We also observed that the frequency of nucleotide misinsertion by pol β was 10–100-fold lower on P-gap-1 compared with the other DNA substrates (Table II).

Singhal and Wilson (17) showed that pol β switches from a distributive to a processive mode of DNA polymerization on short-gapped (2–6 nt) DNA substrates but only if the 5'-margin of the gap is phosphorylated. The very similar effects observed in our processivity experiments using different oligonucleotides (Fig. 2) indicate that this is an intrinsic property of pol β that has no obvious requirement for specific template sequences. Our steady-state kinetic analyses show that the catalytic efficiency and nucleotide insertion fidelity of pol β are also influenced by gap size and 5'-phosphorylation. Moreover, in contrast to what is observed for pol β binding to DNA (18), 5'-phosphorylation is required for both high catalytic efficiency and increased fidelity on 1-nt gapped DNA (Tables I and II). These data extend the model of Prasad et al. (18) by showing that 5'-PO4 residues must mediate a productive catalytic interaction between pol β and DNA even in 1-nt gaps.

The relative low fidelity of pol β observed on the recessed primer-template (f_{ins} = 10^{-3}–10^{-4}, Table II) is comparable with that reported by others on recessed DNA substrates (21, 23, 24). However, our observation of similar fidelities on recessed and P-gap-6 DNAs appears to conflict with recent reports suggesting that pol β is less faithful during 5- and 6-nt gap-filling synthesis (17, 25). This apparent discrepancy may relate to the overall higher catalytic efficiency of pol β on phosphorylated short-gapped DNA (Table I), to template sequence effects, and/or to differences in the assays used to measure fidelity. Additional experiments are required to resolve this. Regardless, our data showing increased fidelity on P-gap-1 DNA indicate that pol β, and by inference BER, may be less error prone than once thought.

Several mechanisms may contribute to the observed effects of gap structure on catalytic efficiency and fidelity. Based on the binding studies of Prasad et al. (18), it appears that the differences in catalytic efficiency on P-gap-1 and gap-1 are not due to differences in the levels of stable DNA binding (at least for pol β-DNA binary complexes detected by cross-linking and competition assays). An attractive general hypothesis is that the 5'-PO4 in a 1-nt gap somehow facilitates formation of a catalytically optimal pol β-DNA complex without affecting overall binding affinity. Amino acid changes at residues distant from the polymerase active site of pol β were recently shown to affect the fidelity of DNA synthesis (19). This indicates that molecular events at the active site respond to long range changes in the pol β protein. Thus, interactions between the DNA 5'-PO4 and the 8-kDa domain of pol β, which also occur at some distance from the active site (7), may remotely alter dNTP binding and/or protein conformational changes required for chemical catalysis (24, 26). Additional kinetic and structural studies will be required to delineate the contribution of these and other mechanisms to pol β substrate recognition and catalytic efficiency. It is particularly germane to examine the role of the 8-kDa domain in directing the interaction of pol β with P-gap-1 DNAs (7, 18).

In summary, we show that purified pol β exhibits relative high catalytic efficiency and fidelity on 5'-phosphorylated 1-nt gapped DNA *in vitro*. This suggests that a primary biochemical function of pol β in the mammalian cell is to fill 5'-phosphorylated 1-nt gaps. Gaps with this structure appear to be requisite intermediates in short-patch BER (1–4) and may exist in other pathways where involvement of pol β is implicated (4, 8–10).

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