Published in final edited form as:

J Mol Biol. 2012 June 8; 419(3-4): 171–182. doi:10.1016/j.jmb.2012.03.013.

Substrate-dependent millisecond domain motions in DNA polymerase β

Rebecca B. Berlow^{2,4}, Monalisa Swain¹, Shibani Dalal³, Joann B. Sweasy^{3,*}, and J. Patrick Loria^{1,2,*}

¹Department of Chemistry, Yale University, 225 Prospect Street, New Haven, CT 06520

²Department of Molecular Biophysics and Biochemistry, 260 Whitney Avenue, Yale University, New Haven, CT

³Department of Therapeutic Radiology and Genetics, Yale University School of Medicine, New Haven, CT 06520

Abstract

DNA polymerase β (Pol β) is a 39 kDa enzyme that performs the vital cellular function of repairing damaged DNA. Mutations in Pol β have been linked to various cancers and these mutations further correlated with altered Pol β enzymatic activity. The fidelity of correct nucleotide incorporation into damaged DNA is essential for Pol β repair function and several studies have implicated conformational changes in Pol β as a determinant of this repair fidelity. In this work, the rate constants for domain motions in Pol β have been determined by solution NMR relaxation dispersion for the apo and substrate, binary forms of Pol β . In apo Pol β , molecular motions, primarily isolated to the DNA lyase domain, are observed to occur at 1400 s⁻¹. Additional analysis suggests that these motions allow apo Pol β to sample a conformation similar to the gapped, DNA substrate bound form. Upon binding DNA, these lyase domain motions are significantly quenched whereas evidence for conformational motions in the polymerase domain become apparent. These NMR studies suggest an alteration in the dynamic landscape of Pol β due to substrate binding. Moreover, a number of the flexible residues identified in this work are also the location of residues, which upon mutation, lead to cancer phenotypes *in vivo*, which may be due to the intimate role of protein motions in Pol β fidelity.

Keywords

DNA Polymerase β ; Solution NMR; Conformational change; Relaxation dispersion; Enzyme motions; Protein dynamics

© 2012 Elsevier Ltd. All rights reserved.

*Corresponding author: patrick.loria@yale.edu, (203)-436-2518; joann.sweasy@yale.edu, (203) 737-2626.

⁴Present Address: Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

M.S. and R.B.B. contributed equally to this work

Accession number

Resonance assignments for apo Pol β were deposited in the BioMagResBank under accession numbers 18267.

Introduction

DNA polymerase β (Pol β) is a 39 kDa, monomeric enzyme and member of the X-family of DNA polymerases. It functions in the base-excision repair (BER) pathway of damaged DNA in a template directed manner. Pol β fills in the nucleotide gaps in double-stranded DNA after DNA glycosylase removes the damaged base and AP endonuclease incises the DNA backbone. Pol β prefers short gapped or single base gapped DNA substrates.¹ *In vivo*, Pol β is confronted with the daunting task of repairing an estimated 20,000 DNA lesions per cell per day and must select the correct nucleotide from the cellular pool of dNTPs.² In accord with its crucial role, mutations in Pol β have been implicated in prostate, cervical, gastric, and colon cancers.³⁻⁸ These mutants have altered DNA repair fidelity that is sequence and dNTP context dependent as well as having altered chemical steps and dNTP binding constants.⁹ Thus, nucleotide selectivity and the fidelity of nucleotide incorporation is an essential aspect of the enzymatic mechanism of Pol β .

In the search for the molecular determinants of dNTP incorporation fidelity, a number of biochemical studies have focused on identifying the rate-limiting step(s) in Pol β catalysis or on monitoring the rates of conformational changes. Nucleotide incorporation into gapped DNA follows an ordered reaction with DNA binding first, followed by Mg^{2+} -dNTP binding. Upon binding the proper dNTP and formation of Watson-Crick pairing with the template strand of the DNA substrate, the phosphodiester bond is formed between the nucleotide and DNA, followed by subsequent release of pyrophosphate (PPi). It has been proposed that chemistry is rate limiting and therefore the step of nucleotidyl transfer could provide the 'fidelity checkpoint' for the overall reaction.^{10; 11} Other studies have suggested that conformational rearrangements (i.e. an 'induced fit' mechanism) in Pol β are also a mechanistic feature designed to ensure proper nucleotide selection and would thus contribute to the fidelity of this enzyme.^{12; 13} The conformational changes that occur during Pol β catalysis have mainly been resolved by X-ray crystallography (Scheme 1).^{12; 14; 15} The three-dimensional structure of Pol β consists of two domains, an 8 kDa amino terminal lyase domain and a 31 kDa polymerase domain. dRP lyase activity is contained within the 8 kDa domain whereas the polymerase domain possesses the catalytic residues for the nucleotidyl transferase function. Upon binding a gapped, double-stranded DNA substrate, the extended lyase domain closes around the DNA (Scheme 1A,B). Additional closure and conformational changes occur in both domains upon binding the dNTP (Scheme 1C,D). With these structures as a basis, numerous transient kinetics experiments have sought to characterize the timescale of these conformational changes.^{10; 16} These experiments have relied primarily upon intrinsic Trp fluorescence or that of an unnatural fluorophore (2-aminopurine), to relate changes in the DNA environment to protein conformational changes, and to subsequently inform on the important structural changes that occur along the enzymatic reaction coordinate.^{17; 18}

Solution NMR methods are well-suited for monitoring conformational changes with atomic resolution, and do not require addition of bulky or potentially perturbing labels. A recent solution NMR study of full length Pol β investigated the sidechain dynamics of ^{13}C -labeled methionine residues and allowed for identification and characterization of some aspects of ligand and metal-induced conformational activation.^{19; 20} We were prompted by those studies and by recently published backbone assignments of a Pol β /DNA complex²¹ to further investigate how motions in Pol β are altered at early stages along the enzyme reaction coordinate. Our results indicate extensive millisecond (ms) motions in apo Pol β , and that these motions are largely quenched in the lyase domain and enhanced in the polymerase domain upon formation of the binary Pol β /DNA/ Mg^{2+} complex.

Results

NMR characterization

The 39 kDa monomeric Pol β was studied by solution NMR spectroscopy. Using TROSY-based triple resonance experiments, 86% of C α , and C β , and 82% of N H for non-proline residues were assigned in the apo complex, which corresponds to approximately 95% of the observable resonances in a ^1H - ^{15}N correlation spectrum. The two-dimensional ^1H - ^{15}N TROSY spectrum²² for the apo enzyme is shown in Figure 1. Backbone assignments were published previously for the isolated lyase subdomain and a portion of the polymerase domain^{23; 24} of the apo enzyme as well as for full-length Pol β bound to a 22-mer gapped DNA substrate.²¹ Many of the assignments presented here for the full-length apo protein are similar to those of the isolated domains, with significant differences near the domain boundaries.

Two magnesium ions (Mg^{2+}) are required for catalytic activity of Pol β . Addition of millimolar quantities of MgCl_2 to our NMR samples did not result in changes in the TROSY spectra. This result was consistent with observations from the $^{13}\text{C}^{\epsilon}$ -Methionine NMR experiments.²⁰ In contrast, formation of the binary complex by addition of a double-stranded, single nucleotide gapped DNA substrate (see Methods) resulted in numerous changes in the TROSY spectrum (SI Figure 1). The response of the amide resonances to binding of the DNA substrate was monitored by NMR titration. Most resonances that shift upon substrate binding appear to be in slow to intermediate chemical exchange (SI Figure 2). In agreement with the nanomolar affinity determined for this substrate,²⁵ saturation of the NMR sample occurred at approximately equimolar concentrations of Pol β and substrate (SI Figure 2). The chemical shift changes observed upon substrate binding are shown in Figure 2. Figure 2C shows the composite $^1\text{H}/^{15}\text{N}$ chemical shift changes after saturation by substrate. Residues with chemical shift changes greater than 1.5 standard deviations from the 10% trimmed mean (0.17 ± 0.07 ppm) are indicated above the horizontal line in Figure 2C and are plotted onto the Pol β /DNA structure in Figure 2D,E. In total there are 27 residues that demonstrate a significant response to DNA binding (Q8, E21, L22, N24, V29, S30, K60, G64, I69, F76, G80, E86, N98, L100, T121, D130, H134, G139, F146, N164, E165, T176, D192, F223, V238, L259, and G268).

The TROSY spectrum of Pol β bound to our DNA substrate is similar to the spectrum of Pol β bound to a 22-mer gapped substrate.²¹ Backbone assignments for substrate-bound Pol β were determined by comparison to the published data, with ambiguities resolved using TROSY-based triple resonance experiments. Six residues are assigned in the apo enzyme but unassigned in the binary complex, presumably due to exchange broadening in the presence of DNA (T10, G14, F25, G105, K141, and K234). In addition, 12 residues are assigned in the binary complex but not in the apo enzyme (D17, K41, G66, R89, T104, R137, Q159, S202, L211, C239, V303, and I323), again likely due to exchange broadening.²¹

Molecular motions in Pol β

^{15}N transverse relaxation rate constants ($R_{2,avg}$) were measured using the relaxation-compensated Carr-Purcell-Meiboom-Gill dispersion experiment.^{26; 27} This experiment measures the average relaxation rate constant ($R_{2,avg}$) of ^{15}N in-phase and anti-phase coherence. At the shortest τ_{cp} time (0.625 ms), the overall mean $R_{2,avg}$ values are 16.4 ± 5.8 s $^{-1}$ and 20.2 ± 7.1 s $^{-1}$ for the apo and binary Pol β enzymes. The increase in $R_{2,avg}$ observed for the binary complex is consistent with a mass increase of 9.6 kDa upon complex formation. Mean $R_{2,avg}$ values were also compared for the individual domains of the enzyme because in the apo enzyme the lyase domain is somewhat isolated from the main body of the

protein (Scheme 1) and could potentially have a different rotational correlation time (and therefore a different transverse relaxation rate constant) than the rest of the enzyme. For apo Pol β , $R_{2,avg} = 15.5 \pm 8.1 \text{ s}^{-1}$ and $17.2 \pm 5.9 \text{ s}^{-1}$ for the 8 kDa lyase domain (res. 1 – 90) and 31 kDa DNA binding domain (res. 91 – 335) respectively, and therefore do not appear to be significantly different from each other. However, the distribution of $R_{2,avg}$ for the lyase domain is skewed with a significant number of residues with $R_{2,avg}$ lower than expected (SI Figure 3). This suggests that some independent rotational diffusion of the lyase domain may occur in the apo enzyme. In the binary complex the lyase and polymerase domains have similar $R_{2,avg}$ values ($21.4 \pm 10.2 \text{ s}^{-1}$ and $19.9 \pm 6.5 \text{ s}^{-1}$, respectively) reflecting the more compact structure in the presence of DNA substrate.

A comparison of $R_{2,avg}$ at $\tau_{cp} = 0.625 \text{ ms}$ ($R_2(1/\tau_{cp})$) versus amino acid sequence shows a rather uniform range of values across the entire protein with several exceptions of elevated transverse relaxation rates noted in Figure 3. For both the apo and binary enzymes a significant number of residues have $R_{2,avg}$ values higher than the mean protein value. These elevated transverse relaxation rates suggest that conformational exchange motions may exist in both the apo enzyme and the binary complex. The details of the elevated transverse relaxation rate constants were explored in more detail by TROSY-CPMG relaxation dispersion experiments, at 600 and 800 MHz on both forms of the enzyme.

CPMG relaxation dispersion experiments

TROSY-based CPMG relaxation dispersion experiments²⁷ were performed at 296 K on apo Pol β at both 14.1 and 18.8 T. Overall, 30 resonances show dispersion profiles characteristic of millisecond (ms) motions (Figure 4). Of these, 8 pairs of resonances (I33,I73; I38,L22; I174,Y271; F275,K141; A70,W325; L19,Y322, A23,I277; and R83,D145) were overlapped in the ^1H - ^{15}N two-dimensional spectrum and therefore the observed motions cannot be assigned to a single residue. Of the resolved resonances, 12 (E21, Y36, A43, I46, D74, F76, A78, E86, K87, F99, E123, D130) dispersion curves were of sufficient quality for fitting only at 800 MHz. For the remaining residues (K27, N28, A47, E75, R102, D116, L122, R126, H135, and F146), relaxation dispersion data at both static fields was further analyzed (Figure 4 and SI Figure 4). The kinetics of the observed motions varied considerably across the enzyme sequence, with k_{ex} values ranging from 600 s^{-1} to $> 4000 \text{ s}^{-1}$. Four residues (N28, A47, H135, and F146) had k_{ex} values $> 4000 \text{ s}^{-1}$ and could not be adequately quantitated using the CPMG dispersion experiment in the absence of other data.²⁸ Thus, for these residues the k_{ex} value represents a lower limit to the kinetics of the exchange process. Individual fits to the dispersion data for the remaining residues with $k_{ex} < 4000 \text{ s}^{-1}$ were all within a factor of two of each other, suggesting that a global model in which k_{ex} is shared by all remaining residues may be a more appropriate description of the data. In the individual fitting procedure, each residue is assumed to have its own k_{ex} and R_{ex} values. In the global model all residues share a single k_{ex} value while R_{ex} remains a residue-specific value. The individual and global models were compared and the P-value from F-tests is 0.77 thus favoring the mathematically simpler, global model. This conclusion is also supported by AIC differences of 44 when comparing the global versus individual model. Therefore, the data for these 18 residues included in the global fitting support a single conformational exchange process with an exchange rate constant equal to $1410 \pm 130 \text{ s}^{-1}$ (Figure 4 and SI Figure 4). For the four residues with k_{ex} values exceeding 4000 s^{-1} , notably, H135 and F146 are in close proximity to each other in the structure of Pol β and are somewhat isolated from the other residues undergoing ms motions (Figure 5). However, N28 and A47 are spatially separate residing on the ends of α -A and α -B, respectively.

For the binary Pol β enzyme (containing 5 mM Mg^{2+} and the single-gapped DNA substrate), significantly fewer residues exhibit dispersion profiles. Only E21 shows dispersion (Figure 6). Analysis of the dispersion data for E21 in the binary enzyme gives k_{ex}

$= 400 \pm 200 \text{ s}^{-1}$ and $R_{ex} = 16.9 \pm 7.8 \text{ s}^{-1}$, compared to values of $1410 \pm 130 \text{ s}^{-1}$ and $3.3 \pm 1.5 \text{ s}^{-1}$ for the apo enzyme respectively. Of the residues that possessed ms motions in the apo enzyme based on the CPMG dispersion experiment, only E21, N28, and D130 have elevated $R_2(1/\tau_{cp})$ values in the binary enzyme. In addition, a number of other residues in the polymerase domain have elevated $R_2(1/\tau_{cp})$ values in the binary complex (Figure 3B) but do not show dispersion in the CPMG experiment (SI Figure 5). This suggests that although conformational motions appear to be present in the substrate-bound enzyme, the kinetics of the motions reside in a time regime to which the CPMG dispersion experiment is insensitive. Several flexible residues in apo lyase domain (T10, G14, F25) were unassigned in the DNA bound enzyme, therefore whether they remain flexible in the bound enzyme is not known. However, other lyase residues including Y36, A43, I46, A47, D74, E75, and F76 are assigned in the DNA bound form and do not show relaxation dispersion profiles nor elevated R_2 values suggesting the motions in the lyase domain is reduced in the binary DNA complex.

Discussion

There have been several NMR dynamics investigations of protein-DNA interactions. These studies, like this one, observed conformational fluctuations in the apo enzyme or DNA binding protein that were suggestive of inherent flexibility that was important for interacting with the DNA. In DNA binding protein PBX homeodomain the C-terminal extension transiently forms, in the absence of DNA, an α -helical segment,²⁹ whereas cAMP binding to CAP facilitates reorientation of the DNA binding domains in this protein.^{30; 31} Similar phenomena were also observed in the DNA repair enzyme, Uracil DNA Glycosylase in which NMR studies suggested the dynamic sampling of alternate enzyme conformations.³² More recently, backbone flexibility in DNA ligase D was suggested to be involved in both activation and inhibition of this enzyme.³³

The apo Pol β enzyme exhibits conformational exchange motions at 22 resolved amide positions. These sites are located primarily in the dRP lyase domain and at the base of the DNA binding site in the polymerase domain (Figure 5). Many of these residues directly contact or are in close proximity to the substrate in the binary complex. All but four of these residues (N28, A47, H135 and F146) have the same k_{ex} value (1400 s^{-1}). These four residues have R_2 values that vary with τ_{cp} in an almost linear fashion indicating significantly elevated exchange rate constants (SI Figure 4). Interestingly, H135 and F146 are somewhat isolated structurally from those with $k_{ex} = 1400 \text{ s}^{-1}$. In contrast, A47 and N28 are both located at opposite ends of the lyase domain and near residues with $k_{ex} = 1400 \text{ s}^{-1}$. Therefore it is not clear why the CPMG dispersion data for these two resonances suggest greatly elevated exchange rate constants.

For the 18 residues with $k_{ex} = 1400 \text{ s}^{-1}$, most appear to be in the fast exchange limit, precluding estimation of equilibrium site populations for this exchange process. For the residues that may be in intermediate to slow exchange, the dispersion data is not of sufficient quality to allow for extraction of populations and $\Delta\omega$ values from non-linear least squares fitting. However, for groups of residues moving in a concerted fashion, the R_{ex} values obtained from dispersion fitting should be linearly related to the ^{15}N chemical shift difference between apo and substrate-bound Pol β ($\Delta\delta^2$)³⁴ if the apo enzyme is in equilibrium with a conformation similar to that when Pol β is bound to DNA. These data are shown in Figure 7. This analysis clearly identifies two clusters of residues. The slope of the line ($p_{apb}\Delta\omega^2/k_{ex}\Delta\delta^2$) for the first cluster of residues (shown in blue) is 3.55×10^{-5} . Assuming that $\Delta\omega = \Delta\delta$, this slope is equivalent to p_{apb}/k_{ex} , allowing estimation of the equilibrium populations, which are 95%:5% for the major and minor conformations. This further suggests that in the apo enzyme the closing rate constant for dRP lyase domain is 70

s^{-1} . This value is similar to prior estimates of the rate constant for domain closure upon nucleotide binding,³⁵ which based on existing models for Pol β function should be distinct from the motion observed by our NMR experiments since dNTP is not included in our samples. This correlation, which suggests the apo enzyme samples the DNA substrate-bound form argues against an induced-fit mechanism, at least with respect to DNA binding. The data points shown in red in Figure 7 (E21, N28, D74, F76, and E86) comprise the second cluster and the equilibrium populations determined from the slope of the fitted line are unrealistic (99.7%:0.3%). It is unlikely that such a highly skewed population distribution would show upward curving relaxation dispersion data. This observation could be due to additional ring current effects from the DNA that would alter $\Delta\delta$ but not $\Delta\omega$ thus invalidating the assumption that $\Delta\omega = \Delta\delta$. Alternatively, these residues could be part of a separate motional process that coincidentally also has an exchange rate constant equal to $1400 s^{-1}$.

In the binary enzyme form only E21 shows a measurable CPMG relaxation dispersion profile with $k_{ex} = 400 s^{-1}$ though there is significant uncertainty in this value. Nonetheless this data suggests a similar time scale for motion as in the apo enzyme. Other residues with conformational exchange motions in the binary enzyme (Figure 3) are largely located in the polymerase domain (A70, D130, G139, Q169, S204, L211, H212, S229, Y296, and T297). This suggests that DNA binding reduces ms motions in the lyase domain relative to the apo enzyme but enhances motions in the polymerase domain (Figure 8). For residues such as G139, S229, Y296, and T297, which are very close to the DNA in the bound form we cannot determine whether the observed flexibility is only present in the bound form or that DNA ring-current effects unmask motions through an increase in the $\Delta\omega$ value. Resolution of this issue will require further characterization. For the remainder of residues distant from the DNA, ring current effects are likely to not be as significant. The motions in the polymerase domain detected only in the binary complex may represent the flexibility in this region necessary for dNTP binding. DNA binding appears to alter the Pol β energy landscape and increase flexibility in additional regions near the dNTP binding site. Whether this increase in motions is necessary to proper selection of dNTP requires additional investigation.

Strikingly, a significant number of the flexible residues identified in these NMR studies correspond to mutations that have been identified in colon cancer tumors and are known to alter Pol β enzymatic activity.³⁶ Mutations of 10 residues in Pol β (N24, G80, D116, R126, H135, G139, E145, S204, S229, and S275) were identified in colorectal tumors and enzymes bearing these mutations were found to be faulty in the DNA BER pathway. Five of these residues show evidence for motions in the binary complex, whereas the remaining five have ms motions in the apo enzyme only. In addition, five other cancer-associated mutants (D74, T79, T121, H134, and E147) reside adjacent to the flexible residues identified in this NMR work. While the data presented here do not demonstrate a causal link between changes in Pol β flexibility and cell transformation, these data suggest that mutation residues that give rise to aberrant Pol β function reside at sites that are undergoing ms motions. Furthermore, the millisecond motions of these cancer-associated residues are ligand dependent, suggesting an important role for ms motions in this enzyme's function.

Materials and methods

Preparation of double stranded 1-bp gapped DNA

DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The DNA sequence was designed as described previously.¹² The DNA sequences corresponding to the template, primer, and downstream oligonucleotide of the gap are 5'-CCGACGGCGCATCAGC-3', 5'-GCTGATGCGC-3', and 5'-pGTCGG-3'. The

downstream oligonucleotide was 5'-phosphorylated. The template, primer, and phosphorylated downstream oligonucleotides (1:1:1) were mixed in 50 mM HEPES, pH 7.4, and 100 mM KCl. The mixture was incubated sequentially at 95°C for 5 min, slowly cooled to 25°C for 2 hours, and immediately transferred to ice. The quality and extent of the annealing was assessed by an 18% native polyacrylamide gel, followed by methylene blue staining.

Expression and Purification of Protein

The tagless DNA construct of rat Pol β was transformed into BL21 DE3 cells. Protein was expressed in the presence of 1 mM IPTG at 20°C overnight. The cells were resuspended in Buffer A (50 mM HEPES, pH 7.4, 100 mM NaCl, 1mM EDTA, 2mM DTT) in the presence of Roche EDTA Free Protease Inhibitor Cocktail and the sample was sonicated for 5×30 seconds on ice, followed by centrifugation at 10,000 RPM at 4°C. The supernatant was filtered through 0.45 micron filter before being loaded onto a HiTrap Heparin column (GE) and separated by a NaCl gradient [Buffer A: 50 mM HEPES pH 7.4, 100 mM NaCl, 1mM EDTA, 2mM DTT; Buffer B: 50 mM HEPES pH 7.4, 2 M NaCl, 1mM EDTA, 2mM DTT] using a fast protein liquid chromatography system (Äkta, GE Healthcare). Fractions containing pure Pol β were identified by electrophoresis on a 10% SDS PAGE gel followed by Coomassie staining, and were pooled, then diluted to 1:10 with Buffer C [50 mM HEPES pH 7.4, 75 mM NaCl, 1mM EDTA, 2mM DTT]. The protein sample was loaded onto a SP Sepharose column (GE), and again separated by a NaCl gradient [Buffer C: 50 mM HEPES pH 7.4, 75 mM NaCl, 1mM EDTA, 2mM DTT; Buffer B: 50 mM HEPES pH 7.4, 2 M NaCl, 1mM EDTA, 2mM DTT]. Fractions containing pure Pol β were identified by 10% SDS PAGE followed by Coomassie blue staining, and were pooled, then concentrated to <1 mL. Finally, the protein was exchanged into buffer D [50 mM HEPES pH 7.4, 100mM KCl, 2mM DTT, 0.02% sodium azide]. The protein was greater than 95% pure as determined by SDS PAGE and Coomassie blue staining. The concentration of the protein was calculated on the basis of an extinction coefficient of $21200 \text{ M}^{-1}\text{cm}^{-1}$ and a molecular weight of 39 kDa. Roche Protease inhibitor cocktail (1:1000 dilution) was added in final NMR sample to prevent degradation.

Pol β samples for NMR assignments were ^2H , ^{13}C , ^{15}N -labeled, whereas samples used for relaxation dispersion experiments were ^2H , ^{15}N -labeled. The Pol β -DNA binary complex was formed by addition of the pre-formed gapped DNA substrate shown below: 5'GCTGATGCGC GTCGG 3' 3'CGACTACGCGGCAGCC 5' to samples of isotopically labeled enzyme. Binding of the gapped DNA was monitored by NMR titration by following resonance shifts upon DNA binding. All enzyme and DNA samples were prepared in buffer containing 50 mM HEPES pH 7.4, 100 mM KCl, 2 mM DTT, 0.02% sodium azide and 7.5% D_2O . The Pol β concentration in the NMR samples was kept below 0.6 mM to avoid aggregation. In addition, prior to formation of the Pol β -DNA binary complex, 5 mM MgCl_2 (also prepared in the NMR buffer) was added to the NMR sample. During addition of both MgCl_2 and DNA, the pH of the NMR sample was monitored after each addition and adjusted if necessary to ensure that all observed changes in the NMR spectra were due to DNA binding. The double-stranded, single-gapped DNA substrate was added in 10 μL increments from a 6 mM stock solution. DNA was added until no further change in NMR resonances was observed. Based on this criteria, saturation occurred at a DNA:Pol β = 1:1.

NMR Experiments

All NMR experiments were performed at 23 °C on Varian Inova spectrometers operating at 600 or 800 MHz at Yale University or at 900 MHz located in the Department of Chemistry and Biochemistry at the University of Colorado-Boulder. The sample temperature was calibrated with a 100% methanol standard prior to each experiment. Backbone assignments

of apo Pol β were determined using data obtained from TROSY versions of the HN(CA)CB, HN(COCA)CB, and HNCA experiments. Backbone assignments of the Pol β binary complex were obtained by comparison to published spectra in combination with triple resonance experiments.²¹

NMR Relaxation Experiments

¹⁵N TROSY-CPMG experiments^{26; 27} were collected with spectral widths of 2500 × 12000 Hz and 256 × 2048 points in the t_1 and t_2 dimensions. The ¹H and ¹⁵N carrier frequencies were set to the water resonance and 120 ppm respectively. The recycle delay for all relaxation experiments was 2.5 s. The CPMG experiments were acquired with a constant relaxation time³⁷ of 40 ms with τ_{cp} delays of 0, 0.625 (x 2), 0.714, 1, 1.25 (x 2), 1.67, 2, 2.5, 3.33, 5 (x 2), and 10 ms. All NMR relaxation data were processed using NMRPipe³⁸ and analyzed using Sparky³⁹ in conjunction with in-house written programs for dispersion fitting. Peak heights were quantified in Sparky using the average of nine points from a 3 × 3 grid centered on the peak maximum. Relaxation rates were determined from peak intensities using in-house written programs. Uncertainties in rates were determined from duplicate measurements and the Jackknife procedure.⁴⁰ Results are reported only for resonances that are not overlapped in the two-dimensional spectra and that have sufficient signal to noise such that reliable quantitation of peak intensities is possible. CPMG relaxation data were analyzed with

$$R_2(1/\tau_{cp}) = R_2^0 + R_{ex} \left[1 - 2 \tanh(k_{ex}\tau_{cp}/2) / (k_{ex}\tau_{cp}) \right]$$

in which $R_{ex} = p_a p_b \Delta\omega^2 / k_{ex}$. The equilibrium populations are given by $p_a(p_b)$, k_{ex} is the sum of the forward and reverse rate constants for a two-site conformational equilibrium and $\Delta\omega$ is the ¹⁵N chemical shift difference between the two conformations, a and b .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

RBB acknowledges support from NIH Biophysical training grant (T32GM008283). J.P.L. acknowledges financial support from the National Institutes of Health (R01 GM099990). J.B.S acknowledges financial support from the National Institutes of Health (R01 CA080830).

Abbreviations

BER	Base excision repair
CPMG	Carr-Purcell-Meiboom-Gill
dNTP	deoxynucleotide triphosphate
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
Pol β	DNA polymerase beta

References

1. Beard WA, Wilson SH. Structure and mechanism of DNA polymerase Beta. Chem Rev. 2006; 106:361–82. [PubMed: 16464010]

2. Barnes DE, Lindahl T. Repair and genetic consequences of endogenous DNA base damage in mammalian cells. *Annu Rev Genet.* 2004; 38:445–76. [PubMed: 15568983]
3. Dalal S, Hile S, Eckert KA, Sun KW, Starcevic D, Sweasy JB. Prostate-cancer-associated I260M variant of DNA polymerase beta is a sequence-specific mutator. *Biochemistry.* 2005; 44:15664–73. [PubMed: 16313169]
4. Dobashi Y, Shuin T, Tsuruga H, Uemura H, Torigoe S, Kubota Y. DNA polymerase beta gene mutation in human prostate cancer. *Cancer Res.* 1994; 54:2827–9. [PubMed: 8187060]
5. Han LP, Qiao YH, Dong ZM, Shi HR, Zhao GQ, Liu D. [Study on DNA polymerase beta gene mutation in human cervical cancer]. *Zhonghua Fu Chan Ke Za Zhi.* 2003; 38:618–20. [PubMed: 14728866]
6. Lang T, Dalal S, Chikova A, DiMaio D, Sweasy JB. The E295K DNA polymerase beta gastric cancer-associated variant interferes with base excision repair and induces cellular transformation. *Mol Cell Biol.* 2007; 27:5587–96. [PubMed: 17526740]
7. Lang T, Maitra M, Starcevic D, Li SX, Sweasy JB. A DNA polymerase beta mutant from colon cancer cells induces mutations. *Proc Natl Acad Sci U S A.* 2004; 101:6074–9. [PubMed: 15075389]
8. Sweasy JB, Lang T, Starcevic D, Sun KW, Lai CC, Dimaio D, Dalal S. Expression of DNA polymerase {beta} cancer-associated variants in mouse cells results in cellular transformation. *Proc Natl Acad Sci U S A.* 2005; 102:14350–5. [PubMed: 16179390]
9. Yamtich J, Sweasy JB. DNA polymerase family X: function, structure, and cellular roles. *Biochim Biophys Acta.* 1804:1136–50.
10. Bakhtina M, Roettger MP, Tsai MD. Contribution of the reverse rate of the conformational step to polymerase beta fidelity. *Biochemistry.* 2009; 48:3197–208. [PubMed: 19231836]
11. Sucato CA, Upton TG, Kashemirov BA, Batra VK, Martinek V, Xiang Y, Beard WA, Pedersen LC, Wilson SH, McKenna CE, Florian J, Warshel A, Goodman MF. Modifying the beta,gamma leaving-group bridging oxygen alters nucleotide incorporation efficiency, fidelity, and the catalytic mechanism of DNA polymerase beta. *Biochemistry.* 2007; 46:461–71. [PubMed: 17209556]
12. Sawaya MR, Prasad R, Wilson SH, Kraut J, Pelletier H. Crystal structures of human DNA polymerase beta complexed with gapped and nicked DNA: evidence for an induced fit mechanism. *Biochemistry.* 1997; 36:11205–15. [PubMed: 9287163]
13. Arora K, Beard WA, Wilson SH, Schlick T. Mismatch-induced conformational distortions in polymerase beta support an induced-fit mechanism for fidelity. *Biochemistry.* 2005; 44:13328–41. [PubMed: 16201758]
14. Pelletier H, Sawaya MR, Kumar A, Wilson SH, Kraut J. Structures of ternary complexes of rat DNA polymerase beta, a DNA template-primer, and ddCTP. *Science.* 1994; 264:1891–903. [PubMed: 7516580]
15. Sawaya MR, Pelletier H, Kumar A, Wilson SH, Kraut J. Crystal structure of rat DNA polymerase beta: evidence for a common polymerase mechanism. *Science.* 1994; 264:1930–5. [PubMed: 7516581]
16. Balbo PB, Wang EC, Tsai MD. Kinetic mechanism of active site assembly and chemical catalysis of DNA polymerase beta. *Biochemistry.* 2011; 50:9865–75. [PubMed: 22010960]
17. Kim SJ, Beard WA, Harvey J, Shock DD, Knutson JR, Wilson SH. Rapid segmental and subdomain motions of DNA polymerase beta. *J Biol Chem.* 2003; 278:5072–81. [PubMed: 12458221]
18. Zhong X, Patel SS, Werneburg BG, Tsai MD. DNA polymerase beta: multiple conformational changes in the mechanism of catalysis. *Biochemistry.* 1997; 36:11891–900. [PubMed: 9305982]
19. Bose-Basu B, DeRose EF, Kirby TW, Mueller GA, Beard WA, Wilson SH, London RE. Dynamic characterization of a DNA repair enzyme: NMR studies of [methyl-13C]methionine-labeled DNA polymerase beta. *Biochemistry.* 2004; 43:8911–22. [PubMed: 15248749]
20. Kirby TW, Derose EF, Cavanaugh NA, Beard WA, Shock DD, Mueller GA, Wilson SH, London RE. Metal-induced DNA translocation leads to DNA polymerase conformational activation. *Nucleic Acids Res.* 2011
21. Mueller GA, DeRose EF, Kirby TW, London RE. NMR assignment of polymerase beta labeled with 2H, 13C, and 15N in complex with substrate DNA. *Biomol NMR Assign.* 2007; 1:33–5. [PubMed: 19421423]

22. Pervushin K, Riek R, Wider G, Wuthrich K. Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc. Natl. Acad. Sci. U S A.* 1997; 94:12366–71. [PubMed: 9356455]
23. Liu D, DeRose EF, Prasad R, Wilson SH, Mullen GP. Assignments of ¹H, ¹⁵N, and ¹³C resonances for the backbone and side chains of the N-terminal domain of DNA polymerase beta. Determination of the secondary structure and tertiary contacts. *Biochemistry.* 1994; 33:9537–45. [PubMed: 8068628]
24. Mullen GP. Solution structure of DNA polymerases and DNA polymerase-substrate complexes. *Methods Enzymol.* 1995; 262:147–71. [PubMed: 8594345]
25. Tsoi PY, Yang M. Kinetic study of various binding modes between human DNA polymerase beta and different DNA substrates by surface-plasmon-resonance biosensor. *Biochem J.* 2002; 361:317–25. [PubMed: 11772403]
26. Loria JP, Rance M, Palmer AG. A Relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy. *J. Am. Chem. Soc.* 1999; 121:2331–2332.
27. Loria JP, Rance M, Palmer AG. A TROSY CPMG sequence for characterizing chemical exchange in large proteins. *J. Biomol. NMR.* 1999; 15:151–155. [PubMed: 10605088]
28. Vallurupalli P, Bouvignies G, Kay LE. Increasing the exchange time-scale that can be probed by CPMG relaxation dispersion NMR. *J Phys Chem B.* 115:14891–900. [PubMed: 22077866]
29. Farber PJ, Mittermaier A. Concerted dynamics link allosteric sites in the PBX homeodomain. *J Mol Biol.* 2010; 405:819–30. [PubMed: 21087615]
30. Passner J, Schultz S, Steitz T. Modeling the cAMP-induced Allosteric Transition Using the Crystal Structure of CAP-cAMP at 2.1 Å Resolution. *Journal Of Molecular Biology.* 2000
31. Tzeng SR, Kalodimos CG. Dynamic activation of an allosteric regulatory protein. *Nature.* 2009; 462:368–72. [PubMed: 19924217]
32. Sun Y, Friedman JI, Stivers JT. Cosolute paramagnetic relaxation enhancements detect transient conformations of human uracil DNA glycosylase (hUNG). *Biochemistry.* 2011; 50:10724–31. [PubMed: 22077282]
33. Natarajan A, Dutta K, Temel DB, Nair PA, Shuman S, Ghose R. Solution structure and DNA-binding properties of the phosphoesterase domain of DNA ligase D. *Nucleic Acids Res.* 2011
34. Massi F, Wang C, Palmer AG 3rd. Solution NMR and computer simulation studies of active site loop motion in triosephosphate isomerase. *Biochemistry.* 2006; 45:10787–94. [PubMed: 16953564]
35. Showalter AK, Lamarche BJ, Bakhtina M, Su MI, Tang KH, Tsai MD. Mechanistic comparison of high-fidelity and error-prone DNA polymerases and ligases involved in DNA repair. *Chem Rev.* 2006; 106:340–60. [PubMed: 16464009]
36. Donigan, KA.; Sun, KW.; Nemec, AA.; Murphy, DL.; Cong, X.; Northrup, V.; Zelterman, D.; Sweasy, JB. The human POLB gene is mutated in a high percentage of colorectal tumors. 2012. Submitted
37. Mulder FA, Skrynnikov NR, Hon B, Dahlquist FW, Kay LE. Measurement of slow (micros-ms) time scale dynamics in protein side chains by (¹⁵N) relaxation dispersion NMR spectroscopy: application to Asn and Gln residues in a cavity mutant of T4 lysozyme. *J. Am. Chem. Soc.* 2001; 123:967–75. [PubMed: 11456632]
38. Delaglio F, Grzesiek S, Vuister G, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR.* 1995; 6:277–293. [PubMed: 8520220]
39. Goddard, T.; Kneller, DG. SPARKY 3. University of California; San Francisco: 1995. SPARKY..
40. Press, WH.; Flannery, BP.; Teukolsky, SA.; Vetterling, WT. The Art of Scientific Computing. 2nd. edit. Cambridge University Press; Cambridge: 1986. Numerical Recipes..

Highlights

- DNA polymerase β (Pol β) repairs short nucleotide gaps in double-stranded DNA.
- Motions in the apo and DNA-bound forms of Pol β are studied by NMR spectroscopy.
- Millisecond motions in the lyase domain of Pol β are abrogated by DNA binding.
- DNA binding enhances flexibility in the polymerase domain of Pol β .
- Increased flexibility of DNA-bound Pol β may be important for nucleotide binding.

Figure 1.

^1H - ^{15}N TROSY correlation spectrum of apo Pol β . Data was acquired at 900 MHz using a triple-resonance cryogenically cooled probe. The Pol β sample was at pH = 7.4 and 296 K. Spectral widths in the t_1 and t_2 dimensions were 3200 Hz and 14500 Hz respectively. The data was acquired with 128 points in the t_1 dimension and 1000 points in the t_2 dimension with 8 scans per t_1 point.

Figure 2.

Chemical shift changes upon DNA binding. Changes in (A) ^1H , (B) ^{15}N , and (C) composite ^1H and ^{15}N chemical shift changes upon saturation with double-stranded, single-gapped DNA in the presence of 5 mM MgCl_2 . In (A) and (B) chemical shift changes represent the difference between shifts measured for the apo enzyme and the binary complex (apo - binary). Composite chemical shift differences in (C) were calculated as

$$\sqrt{\frac{(\delta_{apo}^H - \delta_{binary}^H)^2 + (\delta_{apo}^N - \delta_{binary}^N)^2}{25}}$$
 in which δ is the observed chemical shift value. The horizontal line at $y = 0.28$ represents 1.5σ above the 10% trimmed mean chemical shift change. Select residues with large chemical shift changes are labeled in (C) and those with composite chemical shift changes greater than that indicated by the horizontal bar are shown as blue spheres in (D and E). These residues are shown in blue on two different orientations of the X-ray structure of the binary Pol β complex (PDB ID: 1BPX). The gapped DNA substrate is shown in dark gray.

Figure 3.

^{15}N Transverse relaxation rate constants for Pol β . ^{15}N $R_{2,avg}$ values from a TROSY-relaxation compensated CPMG experiment with $\tau_{cp} = 0.625 \text{ ms}$ are shown for assigned, resolved amino acid residues in (A) apo and (B) binary Pol β . Residues identified on these graphs are additionally shown in Figure 8 mapped onto the three dimensional structure.

Figure 4. Millisecond motions in apo Pol β

CPMG dispersion curves for residues in apo Pol β. The curves represent global fits of equation 1 to the relaxation data, yielding $k_{ex} = 1410$ s⁻¹.

Figure 5. Location of flexible residues

Amino acid residues in apo Pol β undergoing μs – ms motions as determined by ^{15}N CPMG dispersion experiments are shown as spheres on the structure of the apo enzyme (A and B). For comparison, the location of these residues in the DNA-bound conformation are shown in (C and D). Residues with $k_{\text{ex}} = 1410 \text{ s}^{-1}$ are shown in blue and those with $k_{\text{ex}} > 4000 \text{ s}^{-1}$ are colored red. In (C and D), the gapped DNA strand is shown in gray whereas the template strand is colored black. The apo (PDB ID: 1BPD) and DNA-bound (PDB ID: 1BPX) structures were superimposed based on the C-terminal polymerase domain. Select residues are labeled to orient the reader. Depiction of these flexible residues on the bound structure in (C and D) is not meant to imply they are flexible when Pol β is bound to DNA but simply to highlight the positions they occupy when bound to DNA.

Figure 6. Millisecond motions in the binary complex

The CPMG dispersion curve is shown for E21 in the binary Pol β complex. The inset shows the dispersion curve for E21 in the apo enzyme. Relaxation data for both was acquired at 800 MHz.

Figure 7. Evidence for concerted motions in apo Pol β

Residue-specific R_{ex} values from the relaxation dispersion experiments are plotted against the ^{15}N $\Delta\delta^2$ values corresponding to the differences in the ^{15}N chemical shift between apo and DNA bound Pol β . Blue data points have a linear correlation of 0.94. The red data points, while linearly related ($R = 0.65$), do not cluster with the blue data and suggest a separate motional process.

Figure 8. Comparison of ms motions in apo and binary Pol β

Amino acid residues undergoing conformational exchange motions on the ms timescale are depicted on the structures of apo and DNA-bound Pol β. (A) Residues showing CPMG dispersion curves (red) and elevated R_2 values (blue) in the apo enzyme are shown as spheres at the ^{15}N atomic site. (B) Results for the binary Pol β complex are shown in the same manner as in (A). In (B) the bound, gapped DNA molecule is shown with the gapped strand in gray and the templating strand in black.

Scheme 1.

Structures of Pol β along the reaction coordinate. In (A) the open or apo conformation is shown with the N-terminal lyase domain colored dark gray. In (B) the Pol β -gapped-DNA binary complex is shown with the template DNA strand in magenta and the gapped strands in orange. Panel (C) shows the ternary, Pol β -gapped DNA-AMP-(Mg²⁺)₂ with AMP shown as spheres colored using standard atom colors and the two Mg²⁺ ions are shown in cyan spheres. From this view the magnesium ions are ‘in-line’ and therefore only one is visible. Panel (D) is an overlay of panels (B) and (C) with the ternary lyase domain colored black. The DNA is removed for clarity but the two Mg²⁺ ions and nucleotide remain. The C-terminal domains were aligned such that (A) – (D) are shown in the identical orientation.