Structure of Lithocholic Acid Binding to the N-Terminal 8-kDa Domain of DNA Polymerase β[†]

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ABSTRACT: The purpose of this study was to investigate the molecular action of lithocholic acid (LCA), known as a selective inhibitor of DNA polymerase β (pol β). The 39-kDa pol β was separated proteolytically into two fragments of the template−primer binding domain (8 kDa) and the catalytic domain (31 kDa). LCA bound tightly to the 8-kDa fragment but not to the 31-kDa fragment. We examined the structural interaction with the 8-kDa domain using LCA. On ^{1}H-^{15}N HMQC NMR analysis of pol β with LCA, the 8-kDa domain bound to LCA as a 1:1 complex with a dissociation constant (K_D) of 1.56 mM. The chemical shifts were observed only in residues mainly in helix-3, helix-4, and the 79−87 turn of the same face. No significant shifts were observed for helix-1, helix-2, and other loops of the 8-kDa domain. This region was composed mainly of three amino acid residues (Lys60, Leu77, and Thr79) of pol β on the LCA interaction interface. The inhibition mechanism and the structure−function relationship between pol β and LCA is discussed.

The secondary bile acid, lithocholic acid (LCA),[1] is known to promote tumorigenesis in rats induced by the monoalkylating agent, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (*1*, *2*). LCA exerts its activity without further modification by intestinal bacteria, but the molecular mechanism of the cancer-promoting activity by this bile acid long remained obscure (*3*). Recently, Ogawa et al. found that LCA was an inhibitor of mammalian DNA polymerase β (pol β), which is known to be involved in DNA excision repair (*4*). These observations suggest a possible mechanism for tumorigenesis. Therefore, we investigated the precise molecular interaction of LCA and pol β.

We reported previously the mode of biochemical inhibition by long chain fatty acids, potent pol β inhibitors, using two of the pol β fragments that were separated proteolytically (*5*). The fatty acids were found to bind to the 8-kDa DNA-binding domain fragment and to suppress binding to the template-primer DNA. A 10 000-fold higher level of fatty acid was required for binding to the 31-kDa catalytic domain or to inhibit the DNA polymerase activity, suggesting that it directly disturbs the template−primer incorporation into the template−primer binding domain and indirectly competes with the substrate on its binding site in the catalytic domain (*5*). The binding between the enzymes and the fatty acids can be released by detergents without any permanent damage to the structure (*5*, *6*). As described in this paper, the biochemical mode of inhibiton of pol β by LCA was the same as those by the fatty acids, although the chemical structure of LCA greatly differed from those of the fatty acids. Therefore, the three-dimensional structure studies of pol β with or without LCA might be required.

Pol β is the smallest known DNA polymerase in animal cells with a molecular mass of 39 kDa, and its structure is highly conserved among mammals (*7*). This protein has a modular two-domain structure, with apparent flexibility within a protease-sensitive region between residues 82−86, which separates the two domains (*8*, *9*). Treatment with trypsin yields an N-terminal domain fragment (8 kDa), which retains binding affinity for single-stranded DNA (ssDNA) and a C-terminal domain fragment (31 kDa) with reduced DNA polymerase activity (*8*, *9*). We also reported the ^{1}H-^{15}N HMQC NMR mapping results for the pol β 8-kDa domain with or without long chain fatty acids as another group of pol β inhibitors (*10*). The crystal structure of pol β was analyzed (*11*−*16*), and the NMR structure of the N-terminal 8-kDa domain of pol β has been determined (*17*−*19*). In the 8-kDa domain with the fatty acids, the structure that forms the interaction interface included helix-1, helix-2, helix-4, the three turns (residues 1−13, 48−51, and 79−87), and residues adjacent to an Ω-type loop connecting helix-1 and helix-2 of the same face by ^{1}H-^{15}N HMQC NMR analysis (*10*).

In this study, we analyzed the structural interactions of LCA with pol β, especially the N-terminal 8-kDa domain in contact with LCA by NMR and discussed the structure−

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[1] Abbreviations: LCA, lithocholic acid; kDa, kilodaltons; poly(dA), polydeoxyriboadenylic acid; oligo(dT)$_{12-18}$, oligo(12−18) deoxyribothymidylic acid; oligo(dT)$_{16}$, oligo(16) deoxyribothymidylic acid; ssDNA, single stranded DNA.

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function relationship between pol β and LCA in comparison with the NMR mapping results for the pol β with the fatty acids. These studies may help to further clarify the structure and function of pol β and subsequently may allow us to speculate on the in vivo role of DNA polymerase inhibition by LCA and fatty acids.

MATERIALS AND METHODS

Materials. Nucleotides, and chemically synthesized template primers such as poly(dA), oligo(dT)$_{12-18}$, and oligo(dT)$_{16}$ were purchased from Pharmacia (Uppsala, Sweden). [^{3}H]-dTTP (43 Ci/mmol) and [α-^{32}P]-dTTP (3000 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). M13 single-stranded plasmid DNA was purchased from Takara (Tokyo, Japan). Lithocholic acid (LCA) and all other reagents were of analytical grade and were purchased from Wako Ltd. (Osaka, Japan).

Enzymes. Recombinant rat DNA polymerase β (rat pol β) was purified from *Escherichia coli* JMpβ5 as described by Date et al. (*20*). Fragments of 8 and 31 kDa were prepared by controlled proteolysis at 25 °C with trypsin (1500:1, w/w) for 90 min. Fragments were purified as previously described by Kumar et al. (*8*, *9*). The N-terminal 8-kDa fragment of rat pol β (residues 2−87) was overexpressed in *E. coli* strain BL21 harboring the expression plasmid "Lys-87" constructed in our laboratory. Overproduction of the N-terminal 8-kDa domain and the purification procedure were performed essentially as described in our previous paper (*10*).

DNA Polymerase β Assay. Activity of pol β was measured by the methods described previously (*5*, *6*, *21*). Poly(dA)/oligo(dT)$_{12-18}$ and dTTP were used as template−primer DNA and nucleotide substrate for pol β, respectively. LCA was dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s and then added to the reaction mixture. The activity without inhibitor was considered as 100%, and the remaining activity at each inhibitor concentration was determined as a percentage of this value. One unit of pol β activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of deoxyribonucleoside triphosphates (i.e., dTTP) into synthetic template−primers [i.e., poly(dA)/oligo(dT)$_{12-18}$, A/T = 2/1] in 60 min at 37 °C under the normal reaction conditions for the enzyme (*5*, *6*).

Gel Mobility Shift Assay. The gel mobility shift assay was carried out as described by Casas-Finet et al. (*22*). The binding mixture (a final volume of 20 μL) contained 20 mM Tris-HCl, pH 7.5, 40 mM KCl, 50 μg/mL bovine serum albumin (BSA), 10% dimethyl sulfoxide, 2 mM EDTA, 2.2 nmol of M13 plasmid DNA (single-stranded and singly primed), and intact (39-kDa) or 8-kDa domain fragment of purified pol β. Various concentrations of LCA were added to the binding mixture. The mixture was incubated at 25 °C for 30 min. Samples were run on a 1.2% agarose gel in 0.1 M Tris-acetate, pH 8.3, containing 5 mM EDTA at 50 V for 2 h. The agarose gel was stained and detected with ethidium bromide.

In Vitro DNA Synthesis on Poly(dA)/oligo(dT). For DNA synthesis, the reaction mixture (20 μL) contained 50 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 5 mM dithiothreitol, 10% methanol, 20 μM poly(dA)/oligo(dT)$_{16}$ (= 2/1), 20 μM [α-^{32}P]-dTTP (60 Ci/mmol), and intact (39 kDa) or 31-kDa domain fragments of purified pol β. Various concentrations of LCA were dissolved in DMSO and then added to the above reaction mixture. The mixture was then incubated at 37 °C for 10 min. The products were precipitated with 100% ethyl alcohol and then washed with 70% ethyl alcohol. Bromophenol blue (BPB) dye mixture was added to the precipitate, which was then loaded onto a 15% polyacrylamide−7 M urea gel (40 cm × 20 cm × 0.4 mm) in a buffer containing 6.7 mM Tris-borate, pH 7.5, and 1 mM EDTA (*23*). The gel was prerun for 1 h at 2000 V, and electrophoresis was performed at 2000 V. After electrophoresis, the gel was dried and then exposed to imaging plates (IP) for 30 min and scanned with a BAS 2000 Bio Imaging analyzer (Fuji Film, Tokyo).

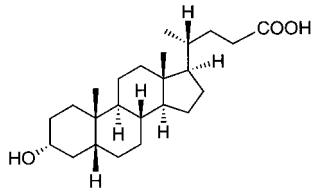

FIGURE 1: Structure of lithocholic acid.

NMR Experiments. NMR spectra were measured at 750 MHz on a Varian Unity-Plus 750 spectrometer. ^{1}H-^{15}N HMQC spectra were recorded at a temperature of 30 °C. Each spectrum was composed of 1024 complex points in the t2 dimension and 96 complex points in t1. The data were zero filled in both dimensions, and a shifted sine-bell was applied as a window function for resolution enhancement. A total of 32 scans per FID were accumulated, leading to a measuring time of 90 min per HMQC spectrum. For ^{15}N-correlated NMR experiments, the N-terminal 8-kDa domain was expressed from BL21/Lys-87 grown on minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source (*10*). In preparing the NMR sample, the purified N-terminal domain was concentrated using a Centricon-3 (Amicon) and exchanged into 5 mM potassium phosphate buffer (pH 7.0) and 20% D_2O. The sample for NMR experiments contained 1.25 mM ^{15}N-labeled N-terminal 8-kDa domain after addition of LCA dissolved in DMSO-d_6.

RESULTS AND DISCUSSION

Effects of Lithocholic Acid on the Activities of Rat DNA Polymerase β. As reported by Ogawa et al., lithocholic acid (LCA) is an inhibitor of eukaryotic DNA polymerases, and among the DNA polymerase α, β, γ, δ, and ϵ, the β-type was most sensitive to inhibition by LCA (*4*). The IC$_{50}$ value of LCA to the β-type was 10 μM. (*4*). The chemical structure of LCA is shown in Figure 1. We have studied the molecular action of LCA using rat DNA polymerase β (pol β). No other DNA metabolic enzymes tested were affected by LCA (data not shown). Therefore, LCA should be classified as an inhibitor of pol β. Here, we report the molecular interaction of LCA with pol β.

To determine the effects of a nonionic detergent on the binding of LCA to pol β, nonidet P-40 (NP-40) was added to the reaction mixture at a concentration of 0.05%. The pol β activity in the absence of LCA was taken as 100%. The inhibitory effect at 10 or 100 μM LCA was largely reversed by adding NP-40 to the reaction mixture (Table 1), indicating that LCA may bind to and interact with the hydrophobic

Table 1: Effects of Detergents on the Inhibition of DNA Polymerase β Activity (0.05 units) by 10 and 100 μM Lithocholic Acid and Carboxyl Ester of Lithocholic Acid[a]

lithocholic acid (LCA) added to the reaction mixture	DNA polymerase β activity (%)
without LCA	100
+ 40 μM poly (rC)	100
+ 80 μg/mL BSA	100
+ 0.05% Nonidet P-40	100
10 μM LCA	50
10 μM LCA + 40 μM poly (rC)	45
10 μM LCA + 80 μg/mL BSA	54
10 μM LCA + 0.05% Nonidet P-40	100
100 μM LCA	2.5
100 μM LCA + 40 μM poly (rC)	4.0
100 μM LCA + 80 μg/mL BSA	3.7
100 μM LCA + 0.05% Nonidet P-40	92
100 μM LCA-ester (carboxyl ester of LCA)	100
100 μM LCA-ester + 40 μM poly (rC)	100
100 μM LCA-ester + 80 μg/mL BSA	100
100 μM LCA-ester + 0.05% Nonidet P-40	100

[a] Forty micromolar poly(rC) and 80 μg/mL BSA or Nonidet P-40 (0.05%) were added to the reaction mixture. In the absence of LCA or LCA-ester, DNA polymerase β activity was taken as 100%.

region on the pol β protein. We also tested whether an excess amount of a substrate analogue, poly(rC) (40 μM), or a protein, BSA (80 μg/mL), could prevent the inhibitory effects of LCA. The purpose of this study was to determine whether the effects of LCA were due to their nonspecific adhesion to the enzyme or to selective binding to specific sites. Poly(rC) and BSA showed little or no influence on the effects of LCA, suggesting that the binding to pol β occurs selectively (Table 1). The thermal transition profiles of double- to single-stranded DNA with or without LCA showed that LCA cannot bind to the DNA molecule (data not shown). Therefore, we analyzed the biochemical and NMR structural relationship between pol β and LCA.

Analysis of Binding between Lithocholic Acid and DNA Polymerase β. Pol β used in this study has been extensively studied, and its amino acid sequence and its secondary and tertiary structures have been reported (*11*−*19*, *24*). The enzyme can be divided into two domain fragments by controlled proteolysis: an 8-kDa N-terminal fragment and a 31-kDa C-terminal fragment (*8*, *9*). The 31-kDa domain is the catalytic part involved in DNA polymerization, and the 8-kDa domain is the template DNA-binding domain. We prepared the whole enzyme of pol β with a molecular mass of 39 kDa and the two domain fragments of 8 kDa and 31 kDa. Both fragments were obtained by controlled proteolysis and purified by FPLC Superose 12 chromatography to near homogeneity (see Figure 4 in ref *5*). The template DNA-binding protein activity and the DNA polymerization activity were analyzed by gel mobility shift assay and by analyzing the products of poly(dA)-oligo(dT)$_{16}$ used as the template−primer, respectively.

Figure 2 shows the results of the gel mobility shift assay of single stranded (ss) M13 plasmid DNA−39 kDa pol β binding complex (lanes 1−4) and M13 DNA−8 kDa domain fragment binding complex (lanes 6−9). In the binding assay, 0.2 nmol of the enzyme or each fragment was added to M13 DNA at a concentration of 2.2 nmol (nucleotide). The 39-kDa pol β and the 8-kDa domain fragment bound to M13 DNA and were shifted in the gel (lanes 1 and 6), but the 31-kDa fragment, the catalytic domain without a DNA binding site, was not (see Figure 5A in ref *5*). LCA interfered with the complex formation between M13 DNA and 39-kDa pol β and between M13 DNA and the 8-kDa fragment to the same extent. The molecular ratios of LCA and the enzyme (or fragment) are shown as inhibitor and enzyme ratios (I/E) in Figure 2. The I/E ratios in lanes 1, 2, 3, and 4 for 39-kDa pol β and lanes 6, 7, 8, and 9 for the 8-kDa domain fragment were 0, 1, 5, and 25, respectively. At a I/E ratio of 5, the interference by LCA was nearly complete, and at a ratio of 1 it disappeared, suggesting that a few molecules, perhaps only one molecule, of LCA competes with each molecule of M13 DNA and subsequently interferes with binding of the 39-kDa pol β or the 8-kDa domain fragment to DNA. LCA appears to directly inhibit the binding of the 8-kDa domain of pol β to the DNA template.

The question thus arose as to whether LCA has any direct influence on the catalytic site. The catalytic 31-kDa domain fragment can bind to the DNA template−primer although weakly and cause polymerization of the DNA. We used poly(dA)-oligo(dT)$_{16}$ as the template−primer and analyzed newly synthesized DNA fragments produced by the 31-kDa protein. In the experiment shown in Figure 3, 25 ng of pol β was used, resulting in the reinitiation of nascent chains during the synthesis period. Figure 3 shows the products formed by the enzyme (lanes 1−4) or the 31-kDa domain fragment (lanes 5 and 6). Pol β is a distributive enzyme (*25*), which adds a single nucleotide and then dissociates from the template−product complex, and the 31-kDa fragment can replicate DNA, as does the whole enzyme. Within a 10-min incubation period, most of the primers were elongated (lanes 4). DNA replication was also observed with 50 ng of the 31-kDa fragment (lane 6). The 8-kDa domain fragment was incapable of replicating DNA (see Figure 6A in ref *5*). At an I/E ratio of more than 5 (lanes 1 and 2), LCA completely suppressed the DNA polymerization by the whole enzyme. At an I/E ratio for the enzyme of 1 (lane 3), DNA synthesis hardly occurred. However, DNA was synthesized even at an I/E ratio for the 31-kDa catalytic fragment of 25 (lane 5). The 31-kDa domain fragment synthesized DNA without interference from LCA. At an I/E ratio of more than 1000, LCA barely inhibited the catalytic activity (data not shown). At the range of the LCA concentrations that influenced the template DNA-binding site on the 8-kDa domain, LCA was thought to indirectly influence DNA polymerization on the 31-kDa catalytic site as a result of a lack of template−primer and to compete with the substrate.

NMR Analysis of Binding of Lithocholic Acid to the N-terminal 8-kDa Domain of DNA Polymerase β. NMR analysis indicated that the 8-kDa domain of pol β (residues 1−87) was formed by four α-helices, packed as two antiparallel pairs. The pairs of α-helices cross one another at 50° giving them a V-like shape. The 8-kDa domain contains a "helix−hairpin-helix" motif (*17*, *18*).

In studying the effect of LCA binding, the recombinant 8-kDa domain fragment was titrated with a 12.5 mM stock solution of LCA. Two-dimensional ^{1}H-^{15}N HMQC spectra were recorded for the 8-kDa domain−LCA complex at LCA concentrations of 0.3125, 0.625, 0.9375, 1.25, 1.5625, 1.875, 2.1875, and 2.5 mM. The complex is in fast exchange on the time-scale of NMR, permitting us to follow the chemical

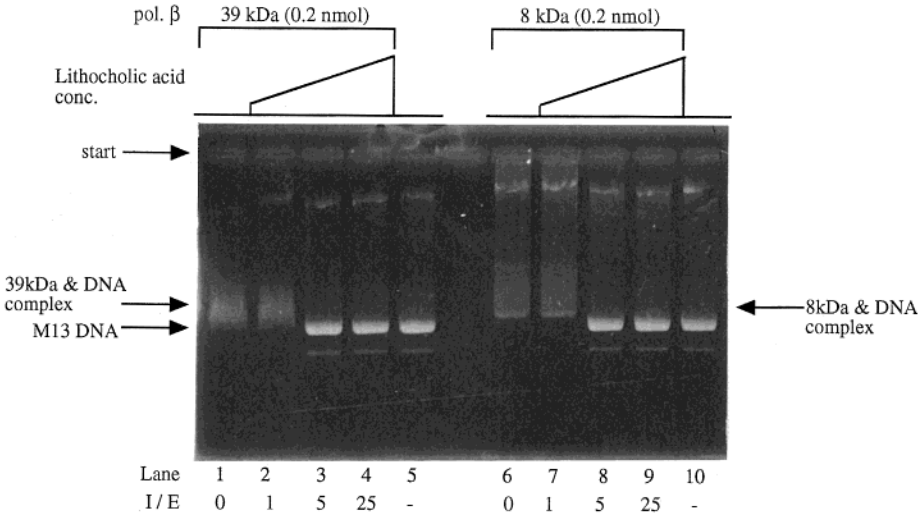

FIGURE 2: Gel mobility shift analysis. Gel shift analysis of binding between M13 single-stranded plasmid DNA and pol β. M13 DNA (2.2 nmol; nucleotide) was mixed with purified proteins and LCA. Lanes 1−4 contained purified pol β (39 kDa) at a concentration of 0.2 nmol; lanes 6−9 contained purified 8-kDa fragment at a concentration of 0.2 nmol; lanes 5 and 10 contained no enzyme. Lanes 1 and 6, lanes 2 and 7, lanes 3 and 8, lanes 4 and 9, and lanes 5 and 10 were mixed with increasing concentrations of LCA: 0, 0.2, 1.0, 5.0, or 0 nmol, respectively. Samples were run on a 1.2% agarose gel in 0.1 M Tris-acetate (pH 8.3) containing 5 mM EDTA at 50 V for 2 h. A photograph of an ethidium bromide-stained gel is shown.

FIGURE 3: Analysis of the poly(dA)/oligo(dT) template/primer synthesis products. DNA synthesis was carried out with 20 μM poly(dA)/oligo(dT)$_{16}$ (= 2/1) and 20 μM of [α-^{32}P]-dTTP (60 Ci/mmol), and the products were examined by gel electrophoresis and imaging analysis as described in Materials and Methods. The enzyme concentrations were as follows: lanes 1−4, 25 ng (0.64 pmol) of the 39-kDa intact pol β; lanes 5 and 6, 50 ng (1.61 pmol) of the 31-kDa fragment. LCA concentrations were as follows: lanes 1−6 were 16, 3.2, 0.64, 0, 40.3, and 0 pmol, respectively. Markers indicate the positions of the extended primer.

shift changes of the backbone NH and ^{15}N signals of the 8-kDa domain upon complex formation by recording a series of ^{1}H-^{15}N HMQC spectra of uniformly ^{15}N-labeled 8-kDa

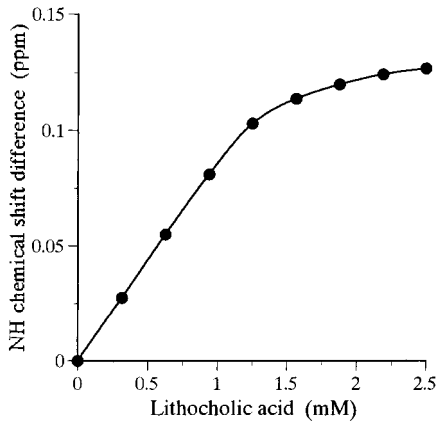

FIGURE 4: Determination of the dissociation constant (K_D) for lithocholic acid binding to the N-terminal 8-kDa domain of DNA polymerase β. Titration of LCA was performed to measure the chemical shift change at the nondegenerate Lys60 amide proton in ^{1}H-^{15}N HMQC NMR. The K_D values of LCA was 1.56 mM.

domain in the presence of increasing amounts of LCA. Of the 79 amides in residues 5−86 of the 8-kDa domain, 75 were assigned in the LCA complex. The cross-peak for Thr79 was sufficiently resolved during titration to allow determination of the mole fraction of protein bound with LCA. The backbone amide of Lys60 displayed the longest chemical shift change on the 8-kDa domain−LCA complex. The change in the chemical shift of Lys60 resonance was interpreted as resulting from the chemical shifts for the free (δF) and the bound forms (δB) of the Lys60 resonance being averaged into a single resonance (δav) [i.e., (δF-δB) $\ll k_{off}$ for the complex (*26*)]. Fitting of the titration curve for the amide proton resonance of Lys60 indicated that the 8-kDa domain binds to LCA as a 1:1 complex with a K_D of 1.56 mM (Figure 4).

Figure 5, panel A, shows the ^{1}H-^{15}N HMQC spectrum of the 8-kDa domain alone (blue contours) overlaid on that of the 1:1 mixture (1.25 mM each) of the 8-kDa domain and LCA (red contours). The NMR mapping result for the 8-kDa domain was indicated in Figure 4A of ref *10*. The data shown

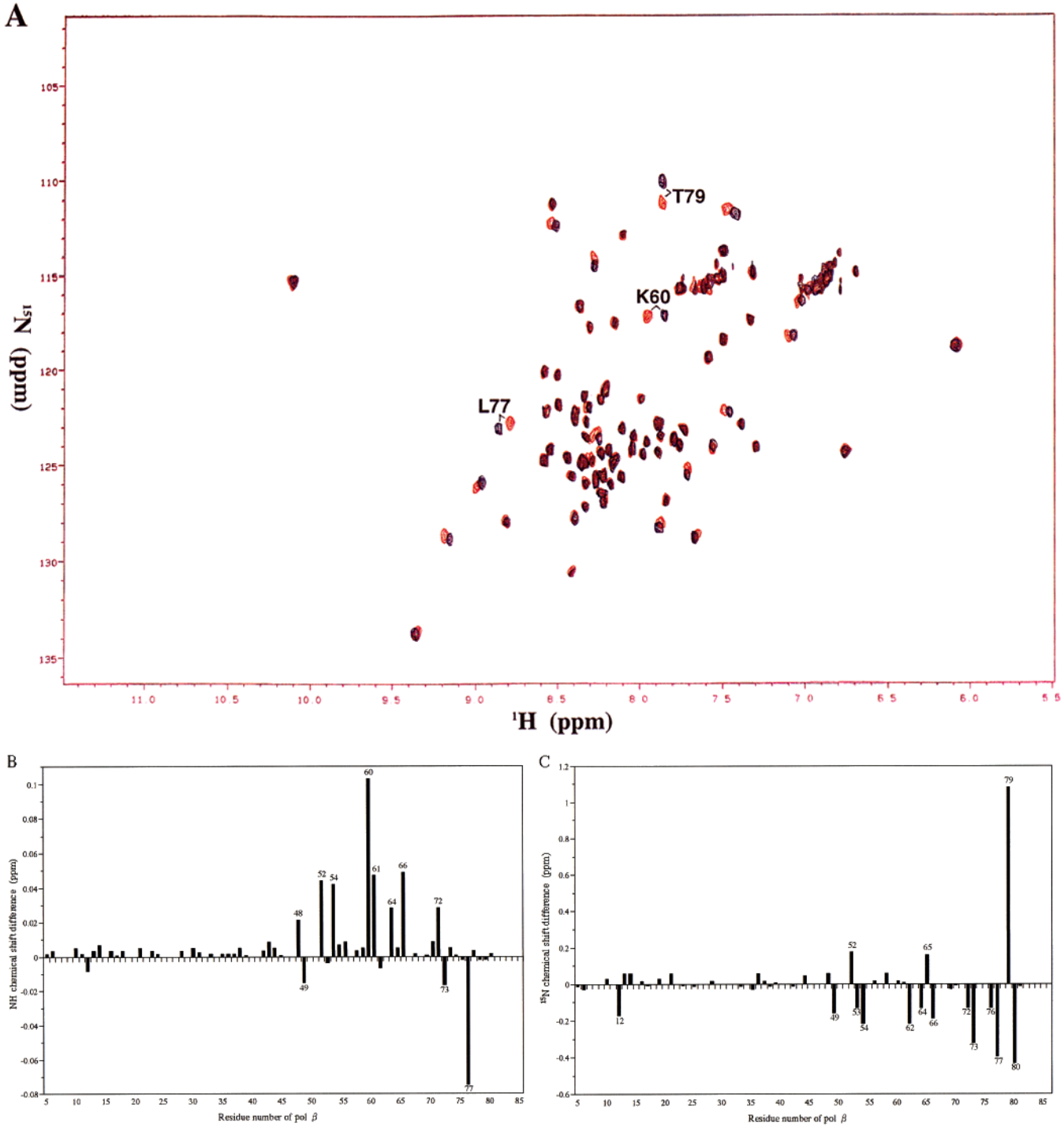

FIGURE 5: Expanded ^{1}H-^{15}N HMQC NMR spectra of the ^{15}N-labeled N-terminal 8-kDa domain-lithocholic acid complex. (A) The N-terminal 8-kDa domain of pol β (1.25 mM) in the absence (blue) or presence (red) of 1.25 mM LCA. The major shifted cross-peaks of the amino acid residues are indicated as the amino acid sequence. (B and C) Chemical shift changes for the N-terminal 8-kDa domain of pol β on complex formation with LCA. The chemical shift differences (the cross-peak shift values of the free domain minus those of the domain complex shown in Figure 4, panel A) for the amide proton chemical shifts (B) or for the amide ^{15}N chemical shifts (C).

in Figure 5, panels B and C, indicate the NH and ^{15}N chemical shift differences in the presence of 1.25 mM LCA along the amino acid sequence of the 8-kDa domain in Figure 5, panel A. Chemical shift changes of $\geq$0.015 for ^{1}H and $\geq$0.1 for ^{15}N were determined for Asn12, Lys48, Tyr49, Lys52, Ile53, Lys54, Lys60, Lys61, Leu62, Gly64, Val65, Gly66, Lys72, Ile73, Phe76, Leu77, Thr79, and Gly80 (Figure 5).

Mapping of Lithocholic Acid Interaction Interface of the N-terminal 8-kDa Domain of DNA Polymerase β. Figure 6, panel A, shows the residues displaying chemical shift changes on binding to LCA in the solution structure of the 8-kDa domain with or without LCA. NH chemical shift changes of 0.015−0.03 ppm and ^{15}N chemical shift changes of 0.1−0.3 ppm (Asn12, Lys48, Tyr49, Ile53, Leu62, Gly64, Val65, Lys72, and Phe76) are shown in yellow. NH chemical shift changes of 0.03−0.06 ppm and ^{15}N chemical shift changes of 0.3−0.6 ppm (Lys52, Lys54, Lys61, Gly66, Ile73, and Gly80) are shown in orange. NH chemical shift changes of more than 0.06 ppm and ^{15}N chemical shift changes of

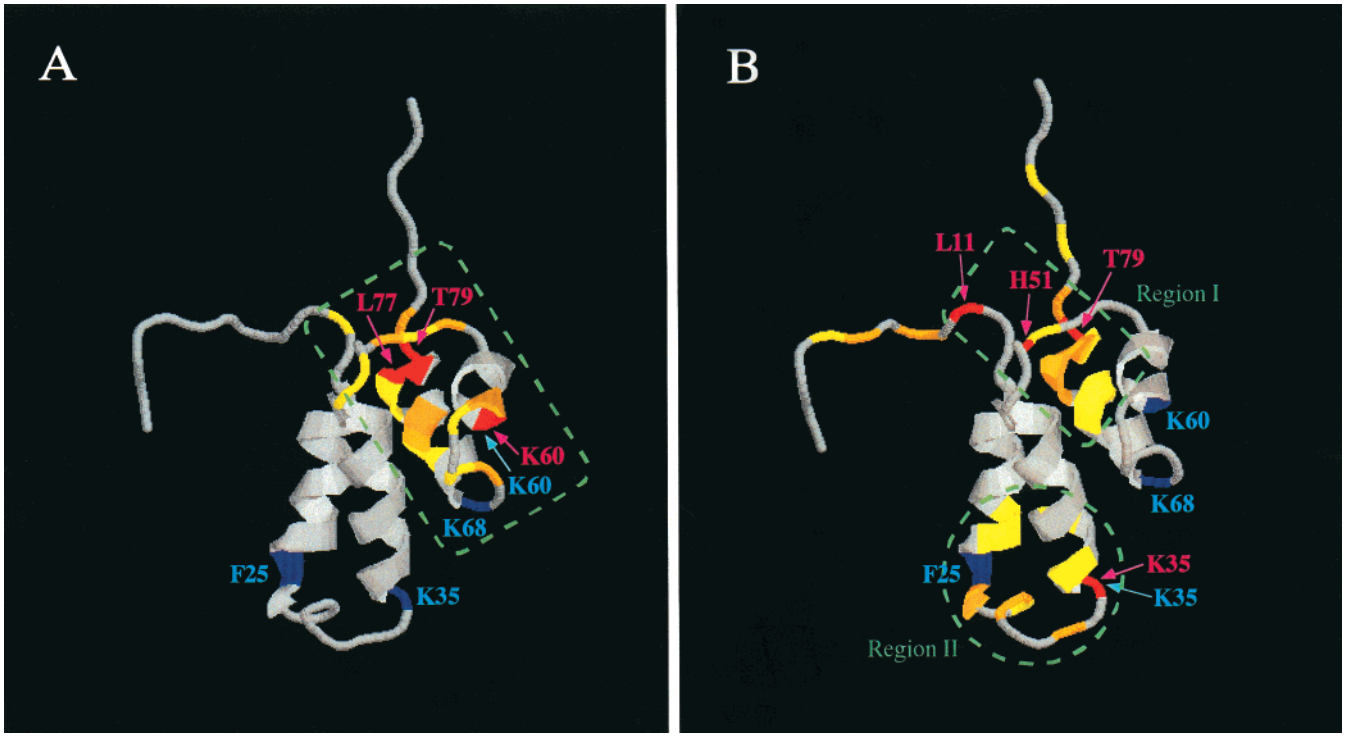

FIGURE 6: Interaction between lithocholic acid and the amino acid residues of the N-terminal 8-kDa domain of rat DNA polymerase β. The N-terminal 8-kDa domain consists of four helices, helix-1 (15−26), helix-2 (36−47), helix-3 (56−61), and helix-4 (69−78), packed tightly to form a hydrophobic core. The remainder of the domain consists of two turns (48−55 and 62−68), an Ω-type loop (27−35), and extended structures (1−14 and 79−87). The Protein Data Base code of the 8-kDa domain of pol β is 1BNO. (A) Interactions between LCA and the 8-kDa domain. The amino acid residues of the major shifted cross-peaks from the ^{1}H-^{15}N HMQC NMR experiments are indicated. 0.015−0.03 ppm of NH chemical shift changes and 0.1−0.3 ppm of ^{15}N chemical shift changes are depicted in yellow. NH chemical shift changes of 0.03−0.06 ppm and ^{15}N chemical shift changes of 0.3−0.6 ppm are indicated in orange. NH chemical shift changes of more than 0.06 ppm and ^{15}N chemical shift changes of more than 0.6 ppm are indicated in red. (B) Interactions between unsaturated long chain fatty acid and the 8-kDa domain. The amino acid residues of the major shifted cross-peaks from ^{1}H-^{15}N HMQC NMR experiments are indicated (*10*). (A and B) Interactions between ssDNA and the 8-kDa domain. The amino acid residues Phe25, Lys35, Lys60, and Lys68, which were shown to be necessary for ssDNA binding activity by site-directed mutagenesis (*19*), are indicated in blue.

more than 0.6 ppm (Lys60, Leu77, and Thr79) are shown in red. In the presence of LCA, the cross-peaks were shifted as follows: Asn12 was in the 1−14 unstructured segment; Lys48, Tyr49, Lys52, Ile53, and Lys54 were in a turn; Lys60 and Lys61 were in helix-3, which is adjacent to the 48−55 turn and the 62−68 turn; Lys72, Ile73, Phe76, and Leu77 were in helix-4, which is adjacent to the 62−68 turn and 79−87 unstructured linker segment; Thr79 and Gly80 were in the unstructured linker segment, which connects to the 31-kDa catalytic domain in the full-length enzyme. These chemical shift changes can be explained in terms of LCA contact and perturbation in the electrostatic charge distribution at the surface. Surface residues displaying chemical shift changes were predominantly, although not entirely, clustered on one side of the domain (Figure 6, panel A). The LCA binding interface of the 8-kDa domain consisted of one region mostly consisting of Lys60 in helix-3, Leu77 in helix-4, and Thr79 in the 79−87 unstructured linker segment (Figure 5 and Figure 6, panel A). LCA must enter the pocket (i.e., the crevice between helix-3 and helix-4) formed by these three amino acids in the 8-kDa domain.

Figure 6, panel A, also shows the mapping in the solution structure of the 8-kDa domain with ssDNA. Prasad et al. reported that template DNA [i.e., p(dT)$_8$] binding activity was impaired in site-directed mutants of Phe25, Lys35, Lys60, or Lys68 (shown blue in Figure 6, panel A) (*19*). The helix-3−hairpin−helix-4 motif and residues in an adjacent Ω-type loop connecting helix-1 and helix-2 form the ssDNA interaction surface (*19*). Since LCA bound to the ssDNA binding region of the 8-kDa domain and competed for binding with template DNA as shown in Figure 2, two of the maps were compared. The only amino acid residue shifted by both LCA binding and ssDNA binding was Lys60 in helix-3 (Figure 6, panel A). In the region shown in Figure 6, panel A, Leu77 and Thr79 are different from the other DNA binding sites (Phe25, Lys35, and Lys68). LCA probably competes with template DNA at residue Lys60 and binds to the site, which subsequently inhibits the ssDNA binding activity on the 8-kDa domain.

As reported previously, unsaturated long chain fatty acids (i.e., linoleic acid or nervonic acid) were also potent inhibitors of pol β and bound competitively to the DNA binding site of the 8-kDa domain (*8*). Figure 6, panel B, shows the residues displaying chemical shift changes on binding to fatty acids in the solution structure of the 8-kDa domain with or without the fatty acids (*10*). Mapping of the amino acids of NH and ^{15}N chemical shift changes was the same as those in Figure 6A in ref *10*. The fatty acid binding interface of the 8-kDa domain consists of two regions: one consisting of Leu11 in the 1−13 unstructured segment, His51 in the 45−55 turn and Thr79 in the 79−87 unstructured linker segment ("region I" in Figure 6, panel B), while the other consists of an Ω-type loop including helix-1 and helix-2 ("region II" in Figure 6, panel B). Figure 6, panel B, also shows mapping in the 8-kDa domain with ssDNA (i.e., Phe25, Lys35, Lys60, and Lys68, shown in blue). As shown

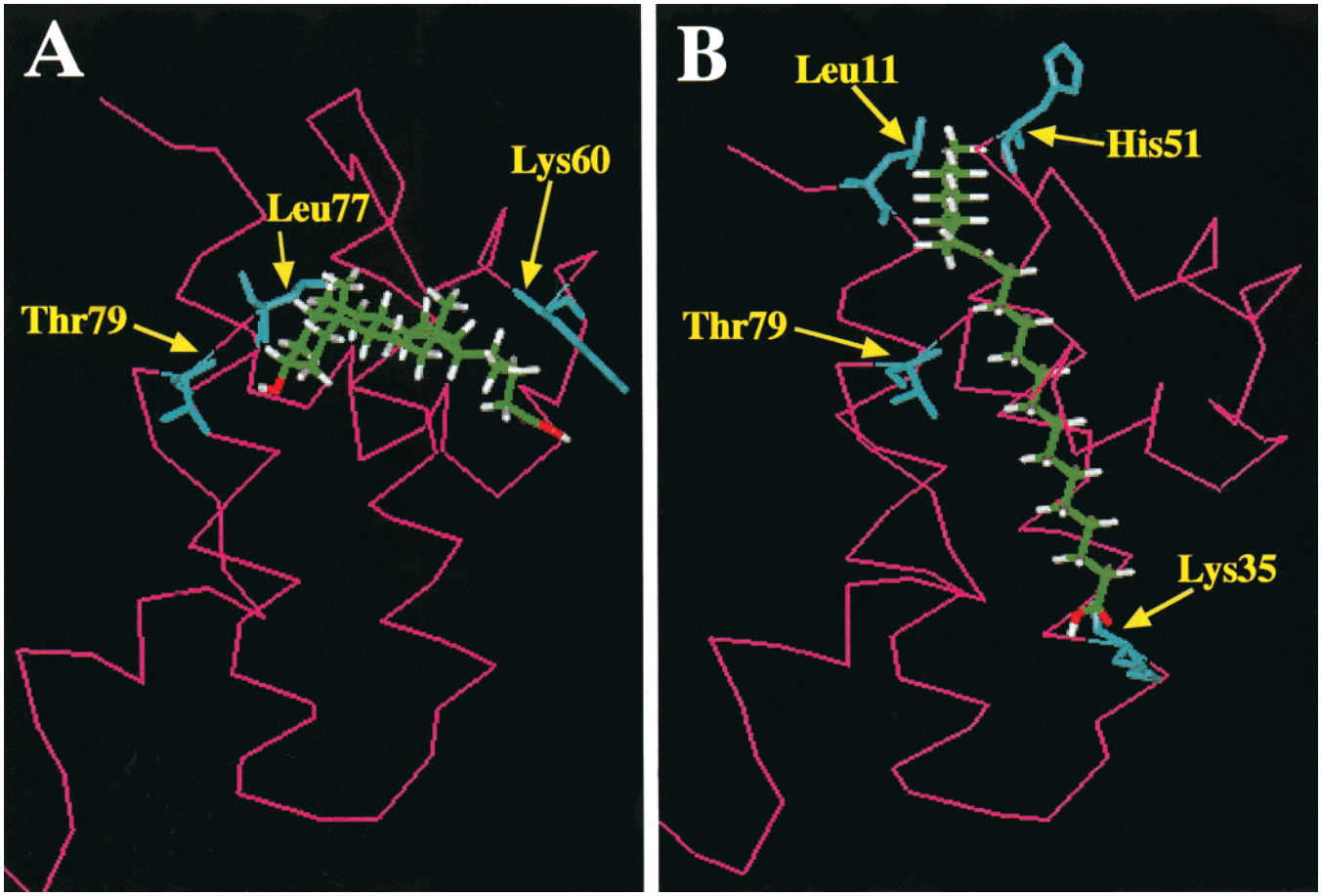

FIGURE 7: Simulation of lithocholic acid interaction interface on the 8-kDa domain of DNA polymerase β. Interactions between LCA (A) and a long chain fatty acid (NA) (B). (A) The amino acid residues Lys60, Leu77, and Thr79, which were significantly shifted as the cross-peaks from ^{1}H-^{15}N HMQC NMR experiments, are depicted in blue. (B) The amino acid residues Leu11, Lys35, His51, and Thr79, which were significantly shifted as the cross-peaks from ^{1}H-^{15}N HMQC NMR experiments, are depicted in blue (*10*). The Cα backbones of the X-ray crystal structures of the 8-kDa domain of pol β is shown in pink. The carbons, oxygens, and hydrogens of the compounds, LCA and NA, were indicated green, red, and white, respectively. The Protein Data Base codes of pol β is 1PBD. This figure was displayed using Insight II/Affinity (Molecular Simulations Inc.).

in Figure 6, panel B, the only site shifted by not only fatty acid binding but also by ssDNA binding was Lys35 in the Ω-type loop including helix-1 and helix-2. Region "II" shown in Figure 6, panel B, appears to have an important role in the fatty acid effect. The fatty acids probably compete with template DNA at residue Lys35 and bind to the site, which subsequently inhibits the ssDNA binding activity on the 8-kDa domain. In region "I" shown in Figure 6, panel B, Leu11, His51, and Thr79 are different from the other DNA binding sites (Phe25, Lys60, and Lys68), suggesting that the methyl end of fatty acids disturbs binding of the template DNA at region "I". Lys35 in region "II" is a hydrophilic amino acid, and Leu11 and His51 in region "I" are hydrophobic. The carboxyl ends of the fatty acids may, therefore, show a preference for binding to the hydrophilic site, and the other side, the methyl end, may be absorbed to the hydrophobic site. The distance between the Lys35 hydrophilic region and the Leu11 and His51 hydrophobic regions fits the length of the U-shaped longer fatty acid.

On the pol β protein, one molecule of LCA competes with one molecule of the template−primer DNA and subsequently interferes with binding of the template−primer to the 8-kDa domain, and the LCA binding indirectly inhibits catalytic activity on the 31-kDa domain. The action of LCA was similar to the effect of the long-chain fatty acids reported previously (*5*, *6*, *10*, *21*), although LCA and the fatty acids are structurally unrelated. Both the hydrophilic end (carboxyl group) and the hydrophobic sites (methyl chain) in the fatty acids played crucial roles in the inhibition (*5*, *6*, *10*). The carboxyl group at carbon position 21 in LCA is thought to be important for the inhibition of pol β, because the compound modified from the carboxyl group to a carboxyl ester could not inhibit the activity (Table 1). The terpenoid rings, i.e., hydrophobic site, of LCA must be required for inhibition.

Modeling of the Lithocholic Acid Interaction Interface on DNA Polymerase β. To confirm the above assumption, modeling of the LCA interaction interface on pol β which was obtained by computer analysis was compared with that of the 8-kDa domain of pol β with a long chain fatty acid, nervonic acid (NA). In this simulation, the fatty acid structures were modeled, but the 8-kDa domain structure was fixed. The results of computer simulation of the binding mode between the 8-kDa domain of pol β (pink line) and LCA is shown in Figure 7, panel A. The computer simulation was performed with Insight II/Affinity (Molecular Simulations Inc., San Diego, USA, 1999). In the ^{1}H-^{15}N HMQC NMR experiments, LCA on the 8-kDa domain was bridged from Lys60, Leu77, and Thr79 and intercalated smoothly into the pocket between helix-3 and helix-4 in the 62−68

turn. In pol β, Lys60 is a hydrophilic amino acid, and Leu77 is hydrophobic. The carboxyl end of LCA may, therefore, show a preference for binding to the hydrophilic residue (i.e., carboxylic acid) of Lys60, and the other side, the terpenoid rings, may be absorbed to the hydrophobic site including the residue of Leu77. Although the residues shown by the three amino acids seemed to be separated from the LCA structure (Figure 7, panel A), the LCA ends are thought to bind to the respective amino acid residues, and at least the Lys60 binding area in the 8-kDa domain peptide must be strained. The unstructured segment of the 79−87 turn is composed of the C-terminal residues and is flexible. Therefore, when LCA bound to the domain at Lys60 and Leu77, the unstructured linker segment including Thr79 appeared to be adjacent to Leu77 in helix-4.

NA on the 8-kDa domain in Figure 7, panel B, was bridged from Lys35 (blue line) to Leu11 (blur line), His51 (blue line), and Thr79 (blue line) and intercalated smoothly into the pocket between helix-1 and helix-2 in the Ω-type loop. Although the residues shown by blue lines seemed to be separated from NA structure (Figure 7), NA ends are thought to bind to the respective amino acid residues, and at least the Lys35 binding area in the 8 kDa domain peptide must be strained. The unstructured segment of the 1−13 turn is composed of the N-terminal residues and is flexible. The 79−87 turn, i.e., the unstructured linker segment, must also be structurally flexible. Therefore, when the fatty acids bound to the domain at His51 and Lys35, the N terminal turn including Leu11 and the unstructured linker segment including Thr79 appear to be adjacent to His51 in the 45−55 turn. The longer chain fatty acids, since they can more likely gain access to region "I", can affect the tighter binding to region "I". The linear chain may not be able to intercalate between helix-1 and helix-2 in the Ω-type loop and thus cannot inhibit pol β activity.

In conclusion, the inhibitory effects of LCA and fatty acids on pol β activity occur by binding between the 8-kDa domain and LCA or the fatty acid as a 1:1 complex. Although the binding to the domain includes the DNA binding amino acid residues, the residues are different between the fatty acid and LCA (i.e., Lys35 for fatty acids and Lys60 for LCA).

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