Mode Analysis of a Fatty Acid Molecule Binding to the N-terminal 8-kDa Domain of DNA Polymerase β

A 1:1 COMPLEX AND BINDING SURFACE

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species, although the biochemical mode of inhibition is the same, and how the fatty acids bind to the N-terminal 8-kDa domain. 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 fatty acids.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The N-terminal 8-kDa fragment of rat DNA pol β (residues 2–87) was overexpressed in *Escherichia 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 have principally been described in our previous report (23). For 15N-correlated NMR experiments, the N-terminal 8-kDa domain was expressed from BL21/Lys-87 grown on minimal medium (degree of 1 nm using up to eight scans. The per residue molar ellipticity at 25 °C. The CD spectra were collected from 260 to 200 nm at a resolution of 1 cm2 dmol−1 cm−1).

**Circular Dichroism Spectroscopy**—For CD analysis, 6.25 μM (50 μg/ml) purified 8-kDa fragments of pol β and 62.5 μM fatty acid mixture (molar ratio, enzyme:inhibitor = 1:10) were dissolved in 5 mM potassium phosphate buffer (pH 7.0) containing 5% methanol. Each spectrum size was 1024 complex with 32P-5'-end-labeled photolabile dT14D, or oromethyl-3μm methyl-3'-deoxy-E-5-[(3 trifluoromethyl-3H-diazirin-3-y1)styryl]uridylate residue at the 3'-terminus, which occupies the catalytic site of the enzyme (12–14). The 8-kDa domain is divided into two domain fragments (8- and 31-kDa polypeptide).

**Analysis of the Binding between Fatty Acids and Fragments of 8 and 31 kDa of Pol β**—The effects of fatty acid were analyzed using proteolytic methods. Rat pol β, which was also used in this study, can be divided into two domain fragments (8- and 31-kDa polypeptides) using controlled proteolysis (12, 13). The 8-kDa domain is the DNA template-binding domain, and the 31-kDa domain is the catalytic part involved in DNA polymerization. According to the methods described by Kumar et al. (12), we purified both of these fragments by fast protein liquid chromatography Superose 12 (lanes 3 and 4 in Fig. 4, Ref. 3) and used them in this experiment. We also suggested previously that the fatty acids bind to pol β and the 8-kDa domain fragment, but not to the 31-kDa domain fragment (3). Both NA and LA appear to interact with the enzyme or the 8-kDa domain fragment in the same way, but the longer chain binds to the domain fragment more tightly and inhibits DNA polymerase activity much more strongly.

**RESULTS AND DISCUSSION**

The in vitro relationship between mammalian DNA polymerases and fatty acids has been investigated (2, 3). As described in the Introduction, longer chain fatty acids (over 18 carbons) strongly inhibited DNA polymerase activities in vitro. Fatty acids in which the carboxyl end is chemically modified can lose the inhibitory effect. In this study, we analyzed the structure of pol β and its relationship to the long-chain fatty acids in more detail. Lineweaver-Burk plots of fatty acids indicated that both the substrate-binding and the template DNA-binding sites of pol β were antagonistically inhibited by fatty acids (2, 3). We tested fatty acids from C18 to the longest commercially available, C34, previously (3). Among the fatty acids examined, the strongest inhibitor was a C24 fatty acid, nervonic acid (NA), and the weakest was a C18 fatty acid, linoleic acid (LA) (3). We therefore analyzed the mode of binding to pol β using the longest and the shortest fatty acids in the present study.

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**Analysis of the Binding between Fatty Acids and Fragments of 8 and 31 kDa of Pol β by Using Cross-linking**—The labeling
abilities of the synthesized $^{32}$P 5'-end-labeled photoprobe (dT$_{14}$D) were investigated using the N-terminal 8-kDa and C-terminal 31-kDa domain fragments of the recombinant rat pol $\beta$ (39 kDa). The $^{32}$P 5'-end-labeled photoprobe (dT$_{14}$D) had a molecular mass of 5 kDa. dT$_{14}$D was mixed with 8- and 31-kDa fragments of pol $\beta$ and irradiated with near-UV light as described under "Experimental Procedures," and then analyzed by gel mobility shift assay (Fig. 1). As shown in Fig. 1A, autoradiography of the radioactive products of the 8-kDa domain fragment resolved by SDS-PAGE showed a shift from the original 8-kDa to the 13 (8 + 5)-kDa position as the labeled protein complex (lane 1). Poly (dT), which is a DNA template, competed with the photoprobe for binding to the 8-kDa domain fragment (lanes 1–3 in Fig. 1A). The 8-kDa band observed in Fig. 1A may have been due to the absorption of very small amount of labeled photoprobe. A small amount of dT$_{14}$D could bind to the 31-kDa domain fragment and cause a shift to the 36 (31 + 5)-kDa position, although this band was faint (lane 1 in Fig. 1A). The SDS-PAGE characteristics of dT$_{14}$D and recombinant rat pol $\beta$ (39 kDa) were shown previously (Fig. 6 of Ref. 25). When the mixture was not irradiated, no cross-linking of the proteins with dT$_{14}$D was observed (lane 4 in Fig. 1A). The 8-kDa domain fragment of pol $\beta$ could be shifted with the cross-linked dT$_{14}$D, but the 31-kDa fragment, the catalytic domain without a DNA-binding site, could not, because this domain has no DNA binding capacity (lane 1 in Fig. 1A). Cross-linking of the fragments of pol $\beta$ with photolabile dT$_{14}$D was inhibited by addition of the natural template, poly(dT) (lanes 1–3 in Fig. 1A), showing that the 8-kDa domain fragment has contained the template DNA-binding site.

LA and NA interfered with complex formation of the 8-kDa fragment and ssDNA template (Fig. 1, B and C). The concentration of 0.8 pmol was added to 18 pmol of the 8-kDa fragment along with various concentrations of LA (Fig. 1B) or NA (Fig. 1C). The I/E (inhibitor/enzyme) ratios in the presence of 0, 1.8, 3.6, and 7.2 $\mu$M fatty acids (LA is Fig. 1B, NA is Fig. 1C) were 0, 0.25, 0.5, and 1, respectively. NA interfered with binding of the DNA to the 8-kDa fragment, and at an I/E ratio of 1, the interference became nearly complete (Fig. 1C). We, therefore, concluded that one molecule of fatty acids competes with oligonucleotide, i.e. dT$_{14}$D, suggesting that a fatty acid molecule interferes with the binding of template DNA to one molecule of the 8-kDa domain fragment. Similar results were obtained using LA instead of NA (Fig. 1B). LA also interfered with DNA binding to the fragment, but the interference was not complete at an I/E ratio of 1. Thus, the C$_{24}$ fatty acid, NA, showed stronger interference than the C$_{18}$ fatty acid, LA, at the I/E ratio of 1. This may explain the observation that the inhibition of pol $\beta$ activity by NA was 10-fold stronger than that by LA although the biochemical mode of inhibition was the same (2, 3).

To explain why the minimum inhibitory dose of the longer chain fatty acid was much lower than that of short chain species, the dissociation constants ($K_D$) between each of the fatty acids and the domain fragment were also analyzed as described in the later part of this report (Fig. 3). To investigate the binding mode including $K_D$ in detail, NMR structures of the N-terminal 8-kDa domain with or without the fatty acids were determined.

CD Spectra of 8-kDa Domain and Mixture of 8-kDa Domain and Fatty Acids—The CD spectra of complexes of the 8-kDa domain fragment and the fatty acids were very similar to the CD spectrum of the 8-kDa domain fragment alone (Fig. 2). The
comparable maximal negative ellipticities at 208 and 220 nm indicated that the overall helical structure in the mixture of the 8-kDa domain, and the fatty acids were similar to that of the 8-kDa domain fragment alone (Fig. 2). The spectra of the protein-LA complex and the protein-NA complex were similar to each other, but the maximal negative ellipticity of 208 nm and the maximal positive ellipticity of 235–260 nm of the protein-NA complex were higher than those of the protein LA complex (Fig. 2). The unchanged ratio of the maximal negative ellipticity at 222 nm versus 208 nm in the mixture of the 8-kDa domain fragment and the fatty acids suggested no increase in helical structure in comparison with the 8-kDa domain fragment alone. On the basis of these results, we concluded that the fatty acids do not adversely affect the overall structure of the 8-kDa domain fragment.

Analysis of the Binding of Fatty Acids to the N-Terminal 8-kDa Domain by NMR—The NMR structures of the N-terminal 8-kDa domain have recently been determined by Wilson, Mullen, and their co-workers (19). According to their results, the 8-kDa domain (residues 1–87) is 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 motif termed the “helix-hairpin-helix.” The protein residues involved in template DNA binding have been identified by NMR using chemical shift changes (16). The helix-3-hairpin-helix-4 motif and residues in an adjacent V-type loop connecting helix-1 and helix-2 form the ssDNA interaction surface (16). Furthermore, they also found that several mutants of the 8-kDa domain (F25W, K35A, K60A, and K68A) showed impaired template DNA binding activity (14). In a biochemical study, using the purified recombinant 8-kDa domain, photochemical cross-linking studies showed that residues Ser-30 and His-34 cross-linked to p(dT)$_{16}$ (27).

In studying the effects of fatty acid binding, the recombinant 8-kDa domain fragment was titrated with a 12.5 mM stock solution of LA or NA. Two-dimensional $^1$H-$^{15}$N HMQC spectra were recorded for the 8-kDa domain-fatty acid complex at fatty acid 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 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 domain in the presence of increasing amounts of fatty acids. Of the 79 amides in residues 5–86 of the 8-kDa domain, 75 amides were assigned in the fatty acid complex. The cross-peak for Leu-11 was sufficiently resolved during the titration to allow determination of the mole fraction of protein bound with fatty acids. The backbone amide of Leu-11 displays the longest chemical shift change upon complexation. The change in the chemical shift of the Leu-11 resonance is interpreted as resulting from the chemical shifts for the free ($\delta_F$) and the bound forms ($\delta_B$) of the

![FIG. 4](image-url)
FIG. 5. Chemical shift changes for the N-terminal 8-kDa domain of DNA polymerase β on complex formation with the fatty acids LA and NA. The chemical shift differences (the cross-peak shift values of the free domain minus those of the domain complex shown in Fig. 4) for the amide proton chemical shifts (A) or for the amide $^{15}$N chemical shifts (B) are shown in bars.
Leu-11 resonance being averaged into a single resonance (δav) (i.e. δF - δB ≪ K_d for the complex (28)). Fitting of the titration curve for the amide proton resonance of Leu-11 indicated that the 8-kDa domain binds to LA or NA as a 1:1 complex with a K_d of 1.02 or 2.64 mM, respectively (Fig. 3), indicating that the longer fatty acid could bind to the fragment more tightly. This probably explains why the minimum inhibitory dose of the longer chain fatty acid was much lower, although the biochemical mode of inhibition was the same. Since C_{16} or shorter fatty acids are expected to have higher K_d values than the K_d of the C_{18} fatty acid, this may also explain why the shorter chain fatty acids cannot inhibit polymerase activity. Fig. 4A shows the 1H-15N HMBC spectrum of the 8-kDa domain alone. Fig. 4B shows the 1H-15N HMBC spectrum of the 8-kDa domain (blue contours) overlaid on that of the 1:1 mixture of the 8-kDa domain and NA (red contours). Similarly, Fig. 4C depicts the superimposed spectra of the 1:1 mixture of the 8-kDa domain and NA (red contours) and the 1:1 mixture of the 8-kDa domain and LA (green contours). Chemical shift changes of ≅0.015 for 1H and ≅0.1 for 15N were determined for Lys-5, Ala-6, Glu-8, Glu-9, Glu-26, Val-29, Ser-30, Ile-33, Lys-35, Asn-37, Tyr-39, His-51, Lys-52, Ile-73, Ala-78, Leu-82, and Lys-84 (Fig. 5). The data in Fig. 5 indicate the NH chemical shift differences in the presence of 1.25 mM LA or NA along the amino acid sequence of the 8-kDa domain in Fig. 4. The shifted cross-peaks for LA were the same as those for NA, except Leu-11 and Thr-79, suggesting that the mode of binding to the domain does not change between LA and NA.

**Mapping of the Fatty Acid Interaction Interface**—Fig. 6A shows the residues displaying chemical shift changes on binding to the fatty acids in the solution structure of the 8-kDa domain with or without fatty acids. NH chemical shift changes of 0.015–0.03 ppm and 15N chemical shift changes of 0.1–0.2 ppm (Lys-5, Leu-22, Ala-23, Asn-28, Asn-37, Tyr-39, Lys-52, Ile-73, Asp-74, Ala-78, Leu-82, and Lys-84) are shown in yellow. NH chemical shift changes of 0.03–0.06 ppm and 15N chemical shift changes of 0.2–0.4 ppm (Ala-6, Gln-8, Glu-9, Glu-26, Val-29, Ser-30, Ile-33, Phe-76, Leu-77, and Gly-80) are shown in orange. NH chemical shift changes of more than 0.06 ppm and 15N chemical shift changes of more than 0.4 ppm (Leu-11, Lys-35, His-51, and Thr-79) are shown in red. These exposed residues showing significant changes were the same between LA and NA (Figs. 4C and 5). In the presence of either LA or NA, the cross-peaks were shifted as follows: Lys-5, Ala-6, Gln-8, Glu-9, and Leu-11 were in the unstructured segment; Glu-26 was in helix-1, which is adjacent to the Ω-type loop; Asn-28, Val-29, Ser-30, Ile-33, and Lys-35 were in the Ω-type loop; Asn-37 and Tyr-39 were in helix-2, which is adjacent to the Ω-type loop; His-51 and Lys-52 were in a turn; Ile-73, Asp-74, Phe-76, Leu-77, and Ala-78 were in helix-4, which is adjacent to the 48–55 turn and 79–87 unstructured linker segment; Thr-79, Gly-80, Leu-82, and Lys-84 were in the unstructured linker segment that connects to the 31-kDa catalytic domain in the full-length enzyme. These chemical shift changes can be explained in terms of the fatty acid 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 (Fig. 6A). Furthermore, the fatty acid-binding interface of the 8-kDa domain consists of two regions: one consisting of Leu-11 in the 1–13 unstructured segment, His-51 in the 45–55 turn, and Thr-79 in the 79–87 unstructured linker segment ("I" in Fig. 6A), while the other consists of an Ω-type loop, including helix-1 and helix-2 ("II" in Fig. 6A). Fig. 6B shows the mapping in the solution structure of the 8-kDa domain with ssDNA. The data determined by Wilson, Mullen, and their co-workers, the NMR structures (16, 19) and the results of site-directed mutagenesis of the 8-kDa domain (14), were used to illustrate the map. According to Prasad et al. (14), the site-directed mutants of Phe-25, Lys-35, Lys-60, or Lys-68 were impaired template DNA binding activity. Since the fatty acids bind to the ssDNA-binding region of the 8-kDa domain and compete for binding with
template DNA as shown in Fig. 1, two of the maps were compared. In Fig. 6B, the residues (Gln-31, Asn-37, Arg-40, Lys-35, His-51, and Thr-79), which were significantly shifted as the cross-peaks from HMQC NMR experiments, are depicted in red. The fatty acids are indicated in yellow. This figure was displayed using Mol Graph (Daikin Industry Ltd.).

FIG. 7. Simulation of the fatty acid interaction interface on the N-terminal 8-kDa domain of rat DNA polymerase β (Protein Data Base code 1BNO). Interactions between the 8-kDa domain and NA (A and B) or LA (C and D) are shown. The x-ray crystal structure of the N-terminal 8-kDa domain of pol β is shown in blue-white. The amino acid residues Leu-11, Lys-35, His-51, and Thr-79, which were significantly shifted as the cross-peaks from HMQC NMR experiments, are depicted in red. The fatty acids are indicated in yellow. This figure was displayed using Mol Graph (Daikin Industry Ltd.).

Modeling of the Fatty Acid Interaction Interface—To confirm the above assumption, we performed modeling analysis using the results of NMR experiments. The results of computer simulation of the binding mode between the N-terminal 8-kDa domain and the fatty acids are shown in Fig. 7. NA (yellow line) on the 8-kDa domain (blue-white line) in Fig. 7, A and B, was bridged from Lys-35 (red line) to Leu-11 (red line), His-51 (red line), and Thr-79 (red line) and intercalated smoothly into the pocket between helix-1 and helix-2 in the Ω-type loop. The distance between the Lys-35 hydrophilic region and Leu-11 and His-51 hydrophobic regions fit the length of U-shaped NA. On the other hand, LA (yellow line) was trapped more deeply in the pocket, although the LA binding model is basically the same as that of NA, and was further away from regions I and II than in the NA binding model (Fig. 7, C and D). The methyl end of LA may not be able to bind firmly to region I, and subsequently, a steeper U-shaped model was postulated for LA (Fig. 7, C and D). In this simulation, the fatty acid structures were modeled, but the 8-kDa domain structure was fixed. Although the residues shown by red lines seemed to be separated from the fatty acid structures (yellow lines) (Fig. 7), the fatty acid ends are
thought to bind to the respective amino acid residues, and at least the Lys-35 binding area in the 8-kDa domain peptide must be strained. The unstructured segment of the 1–13 turn is comprised 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 His-51 and Lys-35, the N-terminal turn including Leu-11 and the unstructured linker segment including Thr-79 appear to be adjacent to His-51 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. LA and SA are present on the membranes. These observations suggest that the inhibitory effect of fatty acids on DNA polymerase activity occur by binding between the 8-kDa domain and the fatty acid molecule is linear and does not form a U-shaped curve as seen in the unsaturated form fatty acids such as LA and NA. 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 lack of an effect of shorter chain fatty acids, the positive relationship between longer carbon chain length and tighter binding, and the configuration effectiveness on pol β can be explained by our model. The inhibitory effects of fatty acids on DNA polymerase activity occur by binding between the 8-kDa domain and the fatty acid as a 1:1 complex, and this binding can be released by nonionic detergents (2, 3). The fatty acids are present on the internal surface of the cytoplasmic membranes. These observations suggest that the inhibitory effect of fatty acids on DNA polymerase activity occurs in vivo and is reversibly controlled by binding to or release of the DNA polymerase from the fatty acids, perhaps on the membranes. These observations may help in determining the mechanisms of control of these enzymes in vivo.

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