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Site-directed mutational analysis of structural interactions of low molecule compounds binding to the N-terminal 8 kDa domain of DNA polymerase β ☆

Shizuka Murakami [a], Shinji Kamisuki [a], Kei-ichi Takata [a], Nobuyuki Kasai [a], Seisuke Kimura [a], Yoshiyuki Mizushina [b], Keisuke Ohta [a], Fumio Sugawara [a], Kengo Sakaguchi [a,*]

[a] *Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba-ken 278-8510, Japan*
[b] *Laboratory of Food and Nutritional Sciences, Department of Nutritional Science, Kobe-Gakuin University, Nishi-ku, Koube-shi, Hyougo-ken 651-2180, Japan*

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Abstract

We previously reported the mode of inhibition of DNA polymerase β (pol. β) by long chain fatty acids and a bile acid, involving binding analyses to the N-terminal 8-kDa DNA binding domain. Here we describe a site-directed mutational analysis in which the key amino acids (L11, K35, H51, K60, L77, and T79), which are direct interaction sites in the domain, were substituted with K, A, A, A, K, and A, respectively. And their pol. β interactions with a C24-long chain fatty acid, nervonic acid (NA), and a bile acid, lithocholic acid (LCA), were investigated by gel mobility shift assay and NMR spectroscopy. In the case of K35A, there was complete loss of DNA binding activity while K60A hardly has any activity. In contrast the other mutations had no appreciable effects. Thus, K35 and K60 are key amino acid sites for binding to template DNA. The DNA binding activities of L11K, H51A, and T79A as well as the wild type were inhibited by NA to the same extent. T79A demonstrated a disturbed interaction with LCA. ^{1}H–^{15}N HSQC NMR analysis indicated that despite their many similarities, the wild-type and the mutant proteins displayed some significant chemical shift differences. Not only were the substituted amino acid residues three-dimensionally shifted, but some amino acids which are positioned far distant from the key amino acids showed a shift. These results suggest that the interaction surface was significantly distorted with the result that LCA could not bind to the domain. These findings confirm our previous biochemical and 3D structural proposals concerning inhibition by NA and LCA.

Keywords: DNA polymerase β; Inhibitor; NMR analysis

Long chain fatty acids with 18 or more carbons in the backbone and the secondary bile acid, lithocholic acid (LCA), can selectively inhibit mammalian DNA polymerases [1–3]. In particular, the *cis*-configurated unsaturated fatty acids such as nervonic acid (NA) are effective at blocking the activity of DNA polymerase β (pol. β) (4,5,6). LCA is known to promote tumorigenesis in rats induced by the monoalkylating agent, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) [7,8], and is also an inhibitor of pol. β [5,6]. We have focused on elucidating the three-dimensional features of binding between pol. β and different types of inhibitors [5,6], and thus using these inhibitors as molecular probes. It is generally impossible to predict the

☆ *Abbreviations:* Pol. β, DNA polymerase β; NA, nervonic acid; LCA, lithocholic acid; ssDNA, single stranded DNA; kDa, kilodaltons.

* Corresponding author. Fax: +81 471 23 9767.
E-mail address: kengo@rs.noda.tus.ac.jp (K. Sakaguchi).

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three-dimensional structure of an enzyme based only on its amino acid sequence. The crystal structure of pol. β has been analyzed in detail [9–16] and the NMR structure of the 8-kDa domain fragment has been determined by Wilson, Mullen, and their co-workers [10–16]. Furthermore, they also found that several mutants of the 8-kDa domain (F25W, K35A, K60A, and K68A) showed impaired template DNA binding activity [13].

Based on the findings, we previously performed a three-dimensional structural model analysis with comparison of the spatial positioning of specific amino acids in pol. β with reference to binding to inhibitors [3–6]. NA and LCA were found to bind to the N-terminal 8-kDa domain of pol. β as 1:1 complexes, but not to the 31-kDa domain [3,4]. In the 8-kDa domain with NA, the structure that forms the interaction interface includes helix-1, helix-2, and helix-4, the three turns (residues 1–13, 48–51, and 79–87) and residues adjacent to the Ω-type loop connecting helix-1 and helix-2 of the same face [3]. No significant shifts were observed for any of the residues on the opposite side of the 8-kDa domain(3). NA closely fits in a rhomboid pocket composed of the four amino acids (L11, K35, H51, and T79) in the 8-kDa domain of pol. β in a manner reminiscent of marquetry. This binding interferes with the binding of template DNA to the site and consequently inhibits the DNA polymerization activity [3,6]. The lack of any effects 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 [1–3].

LCA also tightly binds to the 8-kDa fragment and is a selective inhibitor of pol. β [17]. NMR analysis of the 8-kDa domain in the presence of LCA showed significant shifts only in residues in helix-3, helix-4, and the 79–87 turn of the same face. No significant shifted signals were observed for helix-1, helix-2, and other loops of the 8-kDa domain. This region features three important amino acid residues (K60, L77, and T79) which are on the LCA-interaction interface [4–6].

In order to assess residue-specific contributions to inhibitor-binding to the 8-kDa domain, we performed site-directed mutagenesis and searched for substituted amino acid residues causing NMR chemical shift changes upon binding of inhibitor to the 8-kDa domain. NMR chemical shift mapping effects can result from direct contacts or indirect perturbation and thus indicate overall surfaces of interaction on proteins. This work showed that contrary to expectation with the L11K, K35A, H51A, and T79A mutant proteins, L11 was found not to be involved in the interactions between pol. β 8-kDa and NA.

Based on information available from the NMR structure of the N-terminal 8-kDa domain, we examined its structural interaction with LCA. 1H-15N HSQC NMR analysis of pol. β associated with LCA demonstrated significant chemical shift changes. A triangular pocket formed by three amino acid residues (K60, L77, and T79) of pol. β in the LCA-interaction interface is suggested [5]. Both NA and LCA directly influence the catalytic site. Only T79 was common to the rhomboid and the triangular pockets.

In this study, we conducted studies of mutants of structurally crucial amino acids in the 8-kDa domain in order to prove the predicted three-dimensional structural model experimentally.

Materials and methods

Materials. Nervonic acid (NA), lithocholic acid (LCA) and all other reagents were of analytical grade and purchased from Wako Ltd. (Osaka, Japan).

Sample preparation. Plasmids with a NA- or LCA-resistance gene for site-directed mutagenesis were prepared using the oligodeoxyribonucleotide-directed dual amber method (Site-directed Mutagenesis System Mutant-Super Express Km, TaKaRa Co., Japan). A DNA fragment with a sequence covering the N-terminal 8-kDa of rat DNA polymerase β (pol. β) was built onto the dual amber vector, pKF18k. The PCR products were inserted into suppressor-free *Escherichia coli* MV1184, cultured with kanamycin to obtain transgenic mutants. The mutated sequences from the plasmids were inserted into the expression vector pET28b. The N-terminal 8-kDa fragment of pol. β (residues 1–87) was overexpressed in *E. coli* strain BL21 harboring the expression plasmids constructed above. Overproduction of the N-terminal 8-kDa domain and the purification procedure were performed essentially as described in our previous report [18]. The histidine-tagged proteins were purified via His-resin (Novagen Co.) and Benzamidine Sepharose 4 Fast Flow (high sub) (Amersham Biotech Co.). L11, K35, H51, T79, K60, and L77, the predicted NA- or LCA-binding site amino acids, were changed to K, A, A, A, A, and A, respectively, and the proteins termed L11K, K35A, H51A, T79A, K60A, and L77A.

Protein determination. Protein concentrations were determined by the method of Bradford using γ-globulin as the standard.

Gel mobility shift assay. The gel mobility shift assay was carried out as described by Casas-Finet et al. [19] . The binding mixture (a final volume of 40 μl) contained 20 mM Tris–HCl, pH 7.5, 40 mM KCl, 50 mg/ml bovine serum albumin (BSA), 10% dimethyl sulfoxide, 2 mM EDTA, 2.2 nmol M13 plasmid DNA (single-stranded and singly primed), and 8-kDa domain fragments of purified pol. β or mutant proteins. Various concentrations of NA or LCA were added to the binding mixture, followed by incubation at 25 °C for 60 min. Samples were run on a 1.0% 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 then stained with ethidium bromide.

NMR experiments. For ^{15}N-correlated NMR experiments, the N-terminal 8-kDa domain was expressed from 8 kDa, L11A, K35A, H51A, T79A, and K60A plasmids transformed into BL21(DE3) and grown on minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. In preparing the NMR samples, the purified N-terminal domain was exchanged into 5 mM potassium phosphate buffer (pH 7.0) and 5% D_2O, and concentrated using a Centricon-3 (Amicon). The six samples for NMR experiments contained 800 μM ^{15}N-labeled N-terminal 8-kDa domain or mutant proteins. Fragments of 8-kDa of pol. β were prepared and purified as previously described[3].

NMR spectra were measured at 600 MHz (AVANCE, DRX-600, and BRUKER). ^{1}H–^{15}N HSQC spectra were recorded at a temperature of 30°C. Each spectrum comprised 1024 complex points in the t2 dimension and 128 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 75 min per HSQC spectrum.

Results

Isolation of mutant proteins substituted for structurally crucial amino acids in the 8-kDa domain of DNA polymerase β

Initially, mutants of structurally crucial amino acids in the 8-kDa domain of DNA polymerase β (pol. β) were made. The five amino acids were substituted as shown in Fig. 1. L11, K35, H51, and T79 which are the predicted NA-binding site amino acids were changed to K, A, A, and A, respectively, and the resultant proteins termed L11K, K35A, H51A, and T79A. K60 is one of the binding site amino acids for LCA, is not involved in NA-binding [4,5], and was substituted to A (K60A). The modified nucleotide sequences were inserted into an *E. coli* expression plasmid, and histidine-tagged recombinant L11K, K35A, H51K, K60A, L77K, and T79A proteins produced according to the manual. The solubility of the expressed proteins was very good. Each of the proteins was thoroughly purified using nickel–Sepharose resin and Benzamidine–Sepharose 4B column chromatography, and after concentration was used in the following experiments Fig. 2.

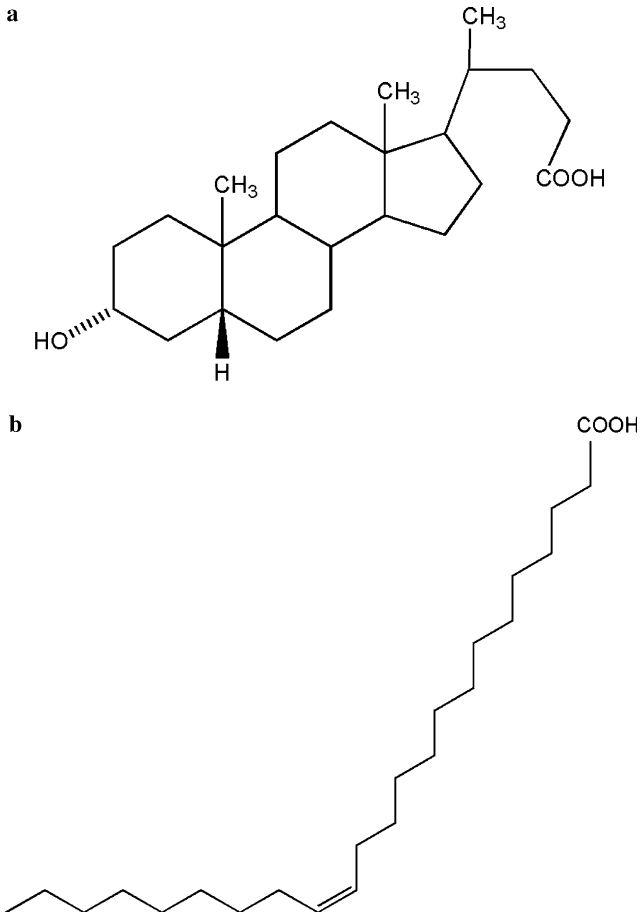

Fig. 1. Molecular structures of the inhibitors (a) lithocholic acid (LCA) (b) nervonic acid (NA) green; carbon atoms, white; hydrogen atoms, red; oxygen atoms.

Binding analysis of the interaction between NA and the mutant 8-kDa domain fragments

Fig. 3 shows the results of the gel mobility shift assay of single-stranded M13 plasmid DNA–8-kDa domain fragment binding complexes, with or without the presence of inhibitors. As shown in Fig. 3(a), neither NA nor LCA bound to the DNA. The findings for L11K, K35A, H51A, and T79A proteins in the absence of inhibitors are shown in Fig. 3(b). In the binding assay, 0.2 nmol of each fragment was added to M13 DNA which was at a concentration of 2.2 nmol (nucleotide). The 8-kDa domain fragment bound to M13 DNA is shifted in the gel (lane 2 in Fig. 3(c)), while the K35A protein demonstrates no binding (lane 5 in Fig. 3(c)).

Since lack of DNA binding activity of the mutant proteins of the 8-kDa domain (F25W, K35A, K60A and K68A) has been reported [14], NA-binding to K35 must be important in the inhibition of activity. Similarly, K60A lost DNA binding activity. The loss of activity may be related to the three-dimensional (3D) structural modification, because heat-denatured 8-kDa domain fragment also lost binding activity (lane 3 in Fig. 3(c)).

As shown in Fig. 3(d), NA interfered with complex formation not only between M13 DNA and the normal 8-kDa fragment (lane 3), but also between the DNA and each of the L11K, H51A and T79A mutants completely (lanes 3–5). In contrast, LCA, which was influential on the triangular pocket formed by K60, L77, and T79, did not strongly affect the DNA binding activity of T79A (lane 3 in Fig. 3(f)), suggesting that the 3D structure of the triangular pocket was hardly changed in the T79A mutant. To confirm this, we tried to obtain a L77-mutated protein, however, we failed in this effort.

Binding analysis of NA and LCA to normal and mutant N-terminal 8-kDa domains by NMR

In order to ensure that the mutations were affecting the structural folding, the folded state of the mutant 8-kDa domain fragment proteins such as L11K, K35A, H51A, T79A, and K60A was determined using two-dimensional (2D) ^{1}H–^{15}N HSQC NMR spectroscopy and the results compared to the normal 8-kDa domain fragment.

In the ^{1}H–^{15}N HSQC spectrum, the skeletal NH group of every residue gives rise to a peak whose position is dependent on the surrounding residues and the conformation of the protein. Therefore, 2D ^{1}H–^{15}N HSQC spectra can be used as a unique fingerprint of the protein. The ^{1}H–^{15}N HSQC spectra of all mutant proteins were found to be well dispersed, reflective of a folded protein, and furthermore, nearly all peak positions were identical or highly similar to those of the normal 8-kDa domain fragment. Shown are representative ^{1}H–^{15}N HSQC spectra for the normal 8-kDa domain fragment and the L11K, K35A, H51A, T79A, and K60A mutant proteins (Fig. 4). In cases where small changes in peak positions were observed

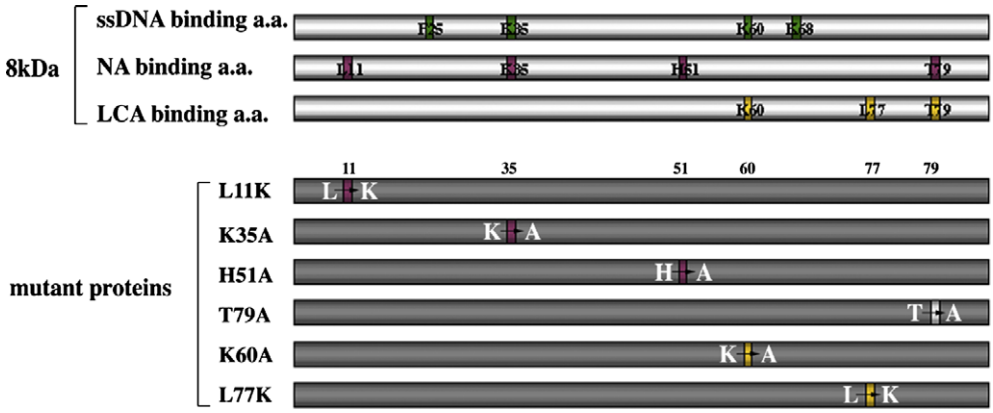

Fig. 2. Amino acid residues of the pol. β–8-kDa domain (PDB code 1DK3) and alignment for the various mutant proteins. The substituted residues are indicated.

for the single site mutations, the differences were localized to the residues in the structure that are in close proximity to the mutation.

Figs. 4(a)–(e) show the ^{1}H–^{15}N HSQC spectra of the normal 8-kDa domain fragment protein (black contours), and the L11K, K35A, H51A, T79A, and K60A proteins (red contours). The spectrum of each of L11K, K35A, H51A, T79A, and K60A was overlaid on that of the normal 8-kDa domain fragment in Figs. 4(a)–(e), respectively.

2D ^{1}H–^{15}N HSQC spectra were recorded for the 8-kDa domain-fatty acid complexes at various concentrations of NA or LCA. The complexes were in fast exchange on the time-scale of NMR, permitting us to follow the proton chemical shift changes of the skeletal NH and ^{15}N signals of the 8-kDa domain upon complex formation. Of the 79 amides in residues 5–86 of the 8-kDa domain, 75 were assigned in the NA or LCA complexes. The cross-peaks for L11 and T79 were sufficiently resolved during the titration to allow determination. The skeletal amide of L11 in the presence of NA displayed the longest chemical shift change upon complex formation. Similarly, that of K60 displayed the longest chemical shift change upon complex formation in the presence of LCA.

The NMR analysis of L11K showed shifts of amino acids, T10 and G13, neighboring to L11 in the peptide sequence, and also of H51, L52, and I53 which are three-dimensionally positioned around L11 (see Fig. 4(a)). In the case of K35A, the contours for amino acids such as A23, I33, N37, and A38, which are sequentially and three-dimensionally close to K35, were greatly shifted (Fig. 4(b)). Interestingly, the mutant protein showed shifts in the peaks of T79, L82, and R83 even though these amino acids are positioned far from K35, both sequentially and three-dimensionally (Fig. 4(b)). The position of K52 was greatly changed in H51A (Fig. 4(c)). In T79A, many amino acids (G56, G60, K72, L77, K81, L82, R83, E26, and K35) were significantly shifted (Fig. 3(d)). G56, G60, K72, L77, K81, L82, and R83 are close to T79 not only in the peptide sequence but also three-dimensionally (Fig. 4(d)). In contrast, the positions of E26 and K35 were changed, even though they are far from T79 in terms of both peptide sequence and three-dimensional structure (Fig. 4(d)). G66 was shifted in all the mutant proteins (see Figs. 4(a)–(e)).

The amino acid residues with ^{1}H chemical shift changes of 0.05 ppm or more, and ^{15}N chemical shift changes of 0.3 ppm or more, were picked up (Fig. 5).

Based on the NMR data, we next focused on chemical shift changes for the 8-kDa domains of pol. β and L11K, K35A, H51A, T79A, and K60A. Chemical shift differences (the cross-peak shift values of the normal domain minus those of the mutant domains) are shown in Fig. 5 for the amide proton chemical shifts or for the amide ^{15}N chemical shift values.

Fig. 6 shows the residues displaying chemical shift changes between the normal and mutant 8-kDa domain proteins. The cross-peaks for L11K were shifted in T10, G13, H51, K52, I53, and G66. T10 and G13 are in the 1–14 unstructured segment; H51, K52, and I53 are in a turn; G66 is in the 62–68 turn (Fig. 6(a)). In the case of K35A, the cross-peaks were moved in A23, S30, Q31, I33, N37, A38, G66, T79, L82, and R83. T79, L82, and R83 are in helix-4 which is adjacent to the 62–68 turn and the 79–87 unstructured linker segment. T79 is in the unstructured linker segment which connects to the 31-kDa catalytic domain in the full-length enzyme (Fig. 6(b)). Similarly, for H51A, amino acids such as G13, K52, and G66 were shifted. G13 is in the 1–14 unstructured segment and K52 in a turn; (Fig. 6(c)). For T79A, amino acids such as E26, S30, Q31, K35, G66, K72, L77, G80, K81, and L82 showed movement. E26, S30, Q31, and K35 are in helix-1; K72 and L77 are in helix-4, which is adjacent to the 62–68 turn and 79–87 unstructured linker segment (Fig. 6(d)). For K60A, amino acids such as Q58, A59, K61, G64, V65, I69, E71, and D74 were also shifted (Fig. 6(e)).

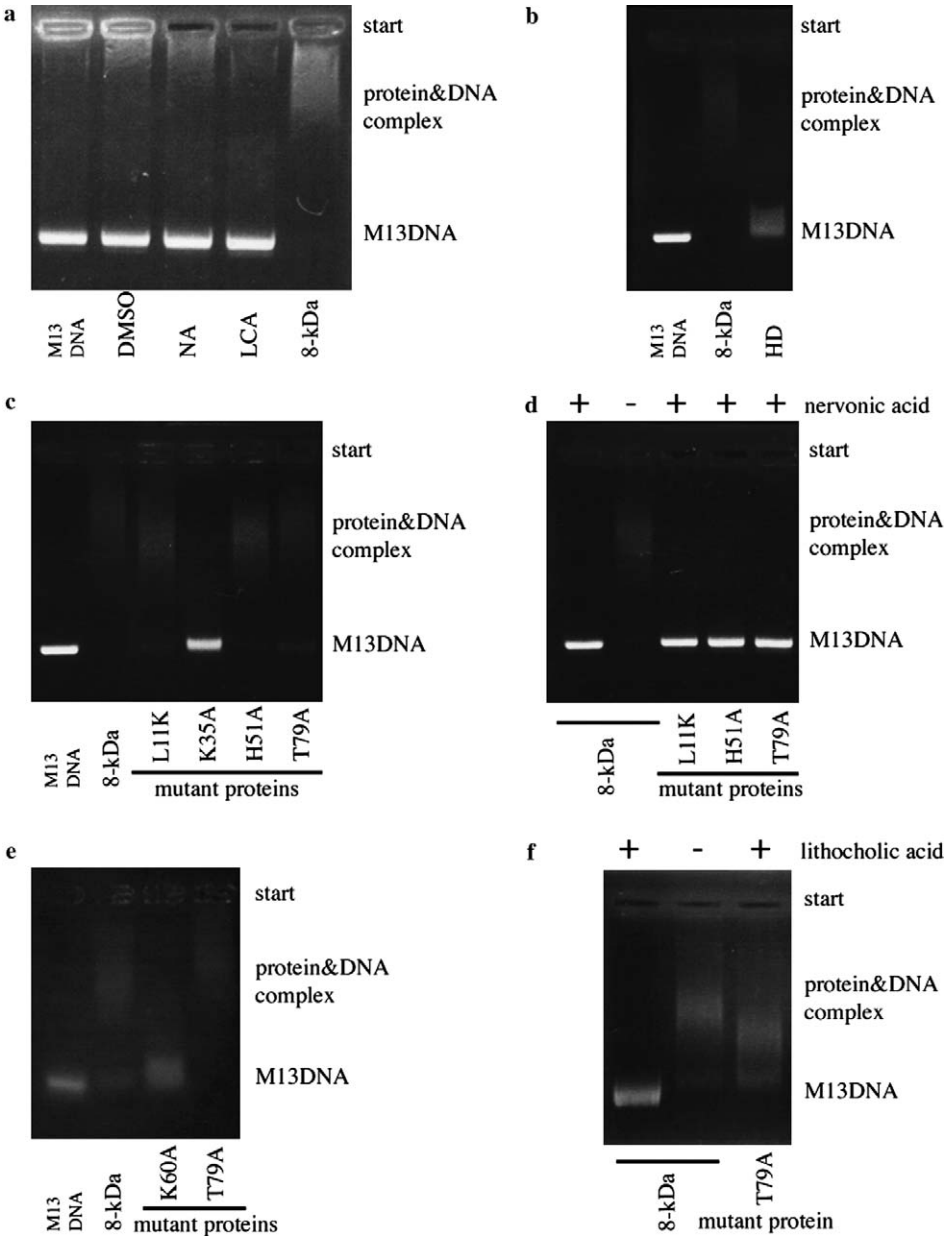

Fig. 3. Agarose gel mobility-shift analysis on 1% (w/v) agarose gel in the presence or absence of inhibitors (NA, LCA). (a) Effects of DMSO or inhibitors on wild-type or 8-kDa fragment binding with M13 DNA: lane 1, none; lane 2, DMSO; lane 3, NA; lane 4, LCA; lane 5, 8-kDa fragment. (b) DNA binding activity of heat denatured fragments was used as a negative control. (c) DNA binding activity of amino acid substituted proteins: lane 1, no protein; lane 2, wild type 8-kDa domain; lane 3, L60K; lane 4, T79A (d) NA at a concentration of 200 μM: lane 1 and lane 2, wild type 8-kDa domain; lane 3, L11K; lane 4, H51A; lane 5; T79A; (e) DNA binding activity of amino acid substituted proteins: lane 1, no protein; lane 2, wild type 8-kDa domain; lane 3, K60A; lane 4, T79A; (f) LCA at a concentration of 50 μM in: lanes 1 and 2; 8-kDa domain, lanes 3; T79A.

Discussion

The purpose of this report was to prove the predicted three-dimensional structural model of the binding of long chain fatty acids and lithocholic acid (LCA) to DNA polymerase β by using mutants of structurally crucial amino acids in the 8-kDa domain. As described previously, in the presence of nervonic acid (NA), the cross-peaks were shifted. The NA-binding interface of the 8-kDa domain consists of two regions; one consisting of H51 in the 45–55 turn and T79 in the 79–87 unstructured linker segment, while the other consists of an Ω-type loop including helix-1 and helix-2 [3]. The data presented here suggest that the Ω-type loop distortion produced by the K35A change in helix 1 directly inhibits the access of template DNA, and that the 1–13 unstructured segment change introduced by L11A and the 45–55 turn change introduced by H51 might greatly distort the rhomboid pocket, and subsequently, give protection from NA-binding.

In the present study, LCA is suggested to compete with template DNA at residue K60, thus inhibiting the ssDNA binding activity of the 8-kDa domain. L77 and T79 are

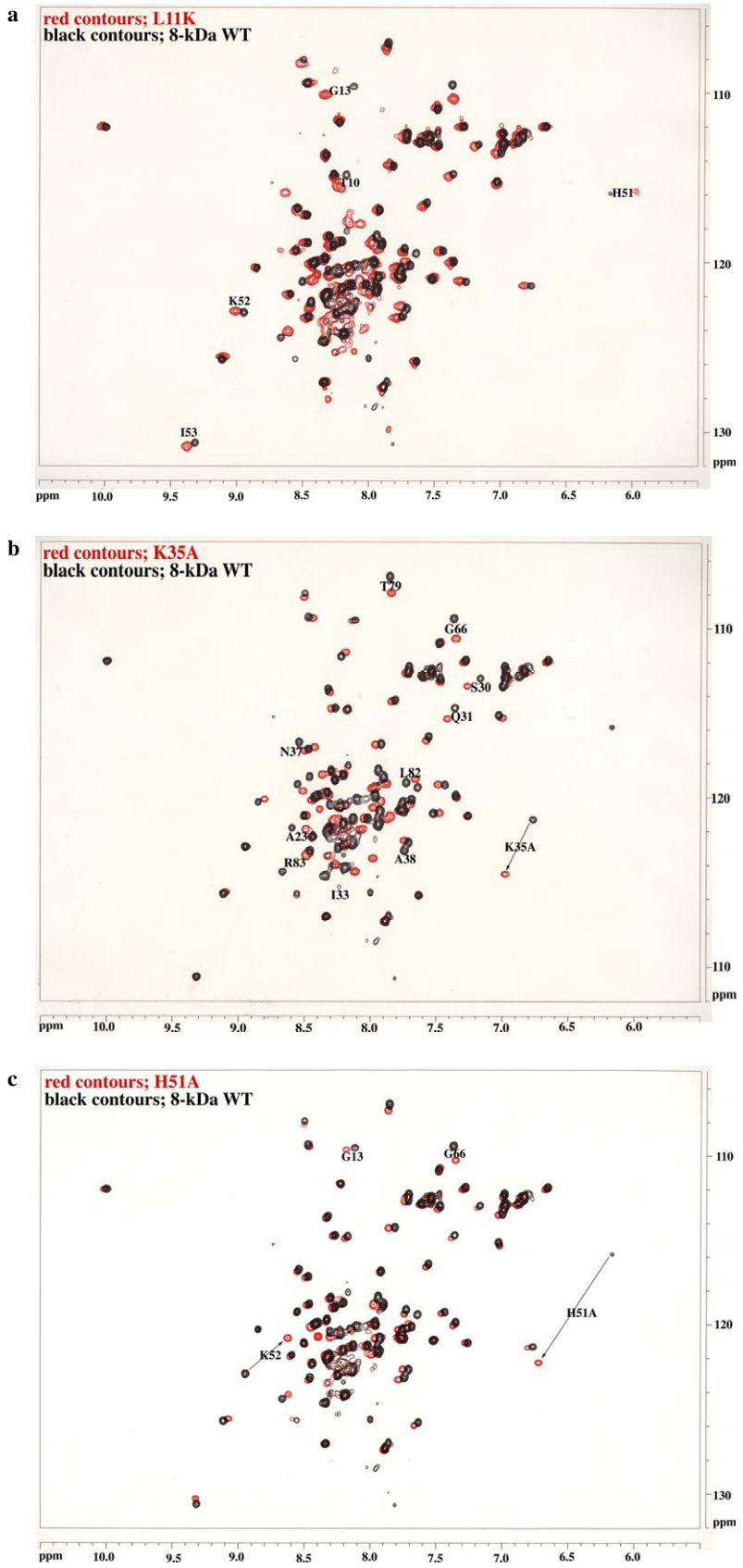

Fig. 4. Expanded ^{1}H–^{15}N HSQC spectra of the 15N-labeled N-terminal 8 kDa domain mutant proteins. The spectrum of wild type DNA pol. β is indicated in black contours and those of the amino acid substituted proteins in red. (a) L11K (b) K35A (c) H51A (d) T79A (e) K60A.

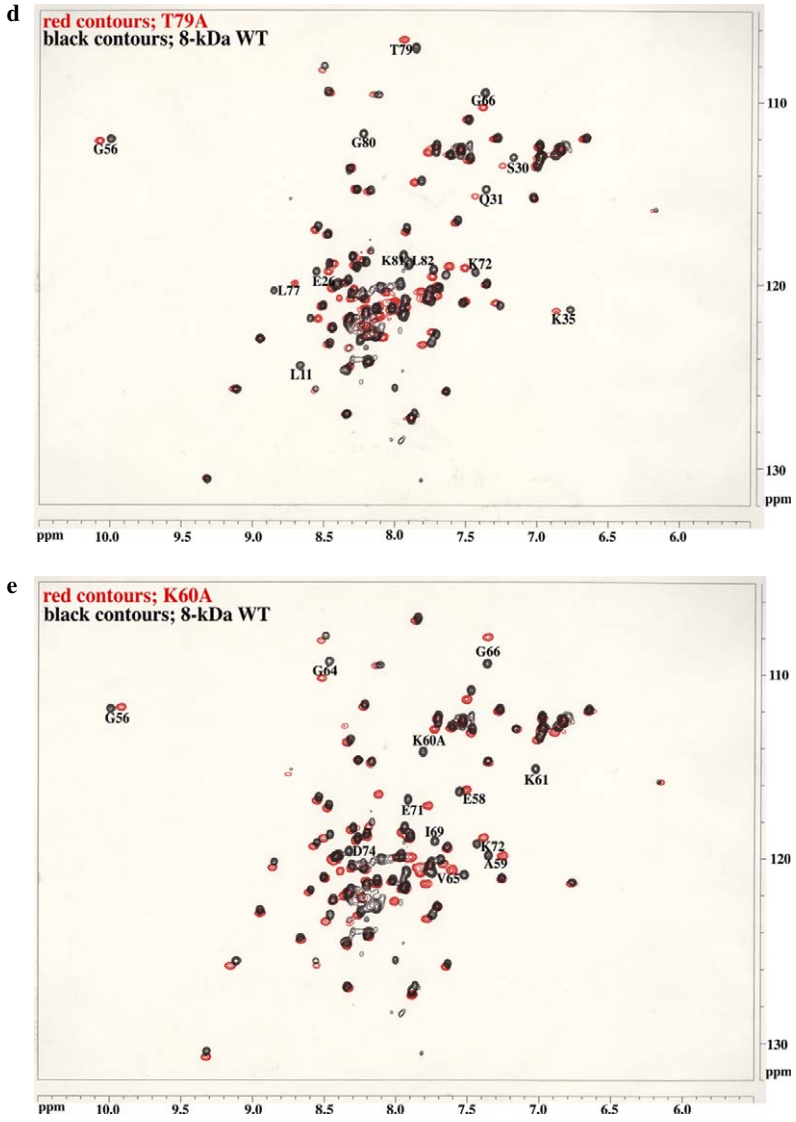

Fig 4. (*continued*)

different from the triangular pocket composed of F25, K35, K60, and K68 [4]. The 3D structural change produced by the T79A mutation may also therefore affect the LCA binding activity of the triangular pocket.

According to Prasad et al. [19], site-directed mutants of F25, K35, K60, or K68 demonstrate impaired template DNA binding activity. Here, the site not only shifted by NA-binding but by ssDNA [i.e., p(dT)8] binding was K35 in the Ω-type loop which includes helix-1 and helix-2. The region "II" appears to have an important role in the fatty acid effect. The fatty acids probably compete with template DNA at residue K35, bind to the site, and thus inhibit the ssDNA binding activity of the 8-kDa domain. In region "I", H51 and T79 are different from the other DNA binding sites (F25, K60, and K68), suggesting that the methyl end of fatty acids disturbs the binding of the template DNA. K35 in region "II" is a hydrophilic amino acid and H51 in region "I" is hydrophobic. The carboxyl ends of the fatty acids may, therefore, show a preference for binding to the hydrophilic site, while the methyl end may be absorbed to the hydrophobic site. The distance between regions "I" and "II" may be a key to explain the characteristics of inhibition by fatty acids.

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]. The present study shows ssDNA binding activity to be lost in K35A, and the chemical shift of T79 residue to be changed. Moreover, although the chemical shift of K35 was altered by the T79A substitution, the mutation was not influential on ssDNA binding activity. Although the structures of the side chains of T and A differ markedly, they are similar with respect to how polar they are. The reduced binding with LCA suggests that the solid three-dimensional structure is more important than the polarity of the amino acid residue side chain.

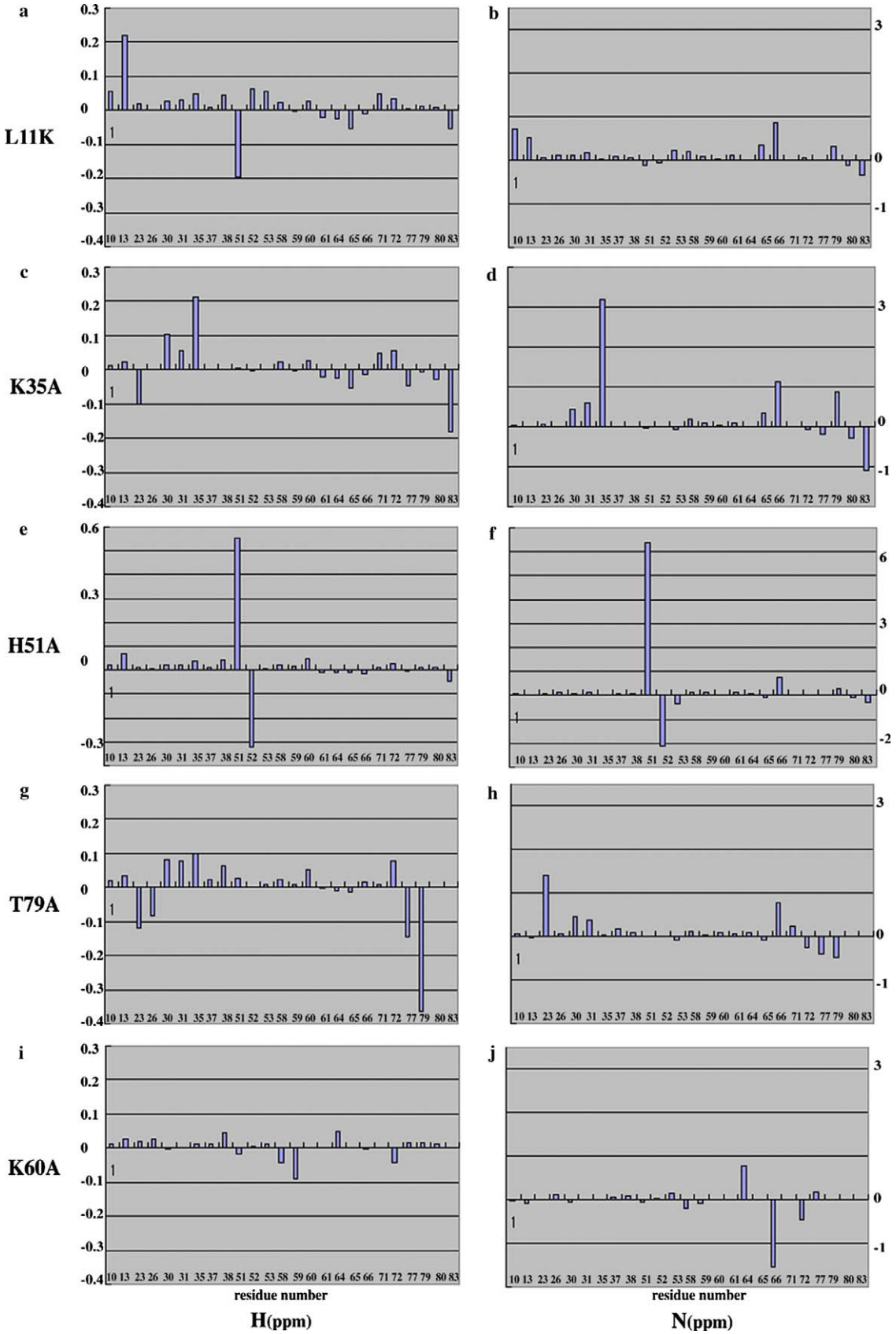

Fig. 5. Chemical shift changes for the mutant DNA polymerase β proteins from ^{1}H–^{15}N HSQC NMR experiments. The difference in chemical shift for amide protons is expressed in terms of mutant minus wild-type. (a,b) L11K (c,d) K35A (e,f) H51A (g,h) T79A (i,j) K60A.

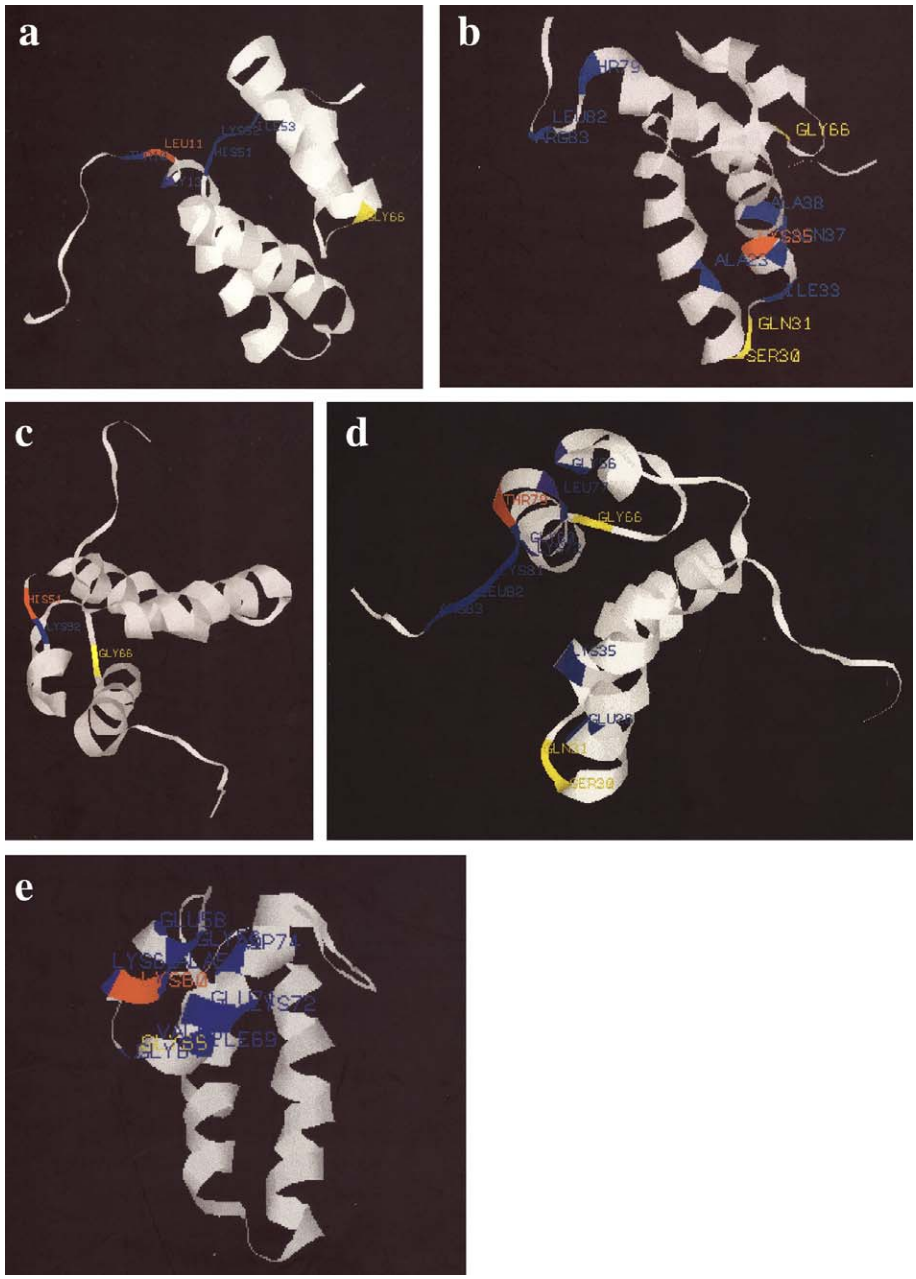

Fig. 6. Chemical shift of amino acid residues of the N-terminal 8-kDa domain of rat DNA polymerase β. The N-terminal domain consists of four helices, helix-1 (15–26), helix-2 (36–47), helix-3 (56–61), and helix-4 (69–78), tightly packed to form a hydrophobic core. The remainder of the domain consists of two turns (48–55 and 62–68), an Ω-type loop (27–35) and extended structures (1–14 and 79–87). The amino acid residues of the major shifted cross-peaks from HSQC NMR experiments are indicated. 0.05 ppm or more of NH chemical shift changes and 0.3 ppm or more of 15N chemical shift changes are depicted. The substituted amino acid is indicated in red. The major shifted residues are indicated in blue. The flexible amino acid residues are indicated in yellow. (a) L11K (b) K35A (c) H51A (d) T79A (e) K60A.

Uninfluential mutant proteins are probably conjectured that the contours indirectly moved by interaction with inhibitors. From the above results, we conclude that for the binding of pol. β–8 kDa domain and LCA, the special structure is more important than the charge and the polar degree of amino acid residues. The domain and LCA bind to the special 3D structure formed by the three amino acid residues K60, L77, and T79. The binding of pol. β–8-kDa domain and NA occurs at amino acid residues L11, K35, H51A, and T79, and two of the amino acid residues (K35 and T79) are essential for DNA binding activity. The data are in agreement with the suggestions described by Sawaya et al. [14] and Mitra et al. [20]. Moreover, for the interaction of pol. β–8-kDa domain and NA, polarity of amino acids is more significant than special filling of amino acids in the groves protein structures. Our results

support the proposition that the affinity between proteins and such compounds depends on the polarity, charge, and three-dimensional structure.

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