Catalytic Center of DNA Polymerase β for Excision of Deoxyribose Phosphate Groups†

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ABSTRACT: The amino-terminal 8-kDa domain of vertebrate DNA polymerase β (pol β) has an activity to excise deoxyribose phosphate (dRP) groups from 5′-incised apurinic/apyrimidinic (AP) sites during base excision repair. The excision reaction proceeds via a β-elimination reaction following formation of a Schiff base between an aldehyde group of the AP site and an amino group of the enzyme. Here we report that the Lys-72 residue of this enzyme is the catalytic center for dRP excision. Substitutions of Lys-72 with Arg or Gln reduced the dRP excision activity to less than 1% of the wild-type 8-kDa domain, while substitutions of Lys-35, Lys-68, or Lys-84 did not abolish its activity. The Lys-72 mutations also significantly decreased Schiff base intermediates trapped by reduction with sodium borohydride. The 8-kDa domain alone was able to bind preferentially to a single-nucleotide gap or 5′-incised synthetic AP site on double-stranded DNA. The Lys-72 mutations did not affect this damage-specific DNA binding activity. When introduced into the intact enzyme, a mutation of Lys-72 to Arg did not affect DNA synthesis activity of pol β, but eliminated the repair activity. Addition of the wild-type 8-kDa domain to this reaction restored the repair activity. These results indicate a specific role of Lys-72 of pol β in the dRP excision during base excision repair.

Base excision repair is the major mechanism to correct modified bases, AP sites,[1] and single-strand DNA breaks (*1*). Various types of modified bases are recognized and removed by DNA-*N*-glycosylases specific for each damaged structure, resulting in a DNA with an AP site, which is a common intermediate product in base excision repair. The subsequent repair of AP sites proceeds through four sequential steps: incision of the AP site at its 5′ side, excision of a dRP group, DNA synthesis, and ligation. These four steps can be accomplished by two pathways. One pathway employs three enzymes in vitro: AP endonuclease, pol β, and DNA ligase (*2*); another pathway employs a different set of protein factors including proliferating cell nuclear antigen (PCNA) (*3, 4, 5*). Among the three enzymes involved in the first pathway, pol β catalyzes two separate reactions: the dRP excision and the DNA synthesis (*2*).

The active site of pol β for the dRP excision resides in the amino-terminal 8-kDa domain. The recombinant 8-kDa domain is able to exert this reaction as efficiently as the intact 39-kDa enzyme. In the excision step, pol β releases an unsaturated derivative of dRP, indicating that the reaction proceeds by β-elimination (*2*). There are many proteins reported to catalyze β-elimination at AP sites, including a number of DNA-*N*-glycosylase-associated AP lyases and histones (*6, 7*). Small molecules such as polyamines and tripeptides, Lys-Trp-Lys and Lys-Tyr-Lys, are also known as AP site-specific β-elimination catalysts (*6, 8, 9*). The 8-kDa domain derived from pol β is unique: the size of the 8-kDa domain (87 amino acids) is much smaller in comparison to DNA-*N*-glycosylase/AP lyases; its specific activity for dRP excision is much higher than those of histones, polyamines, and tripeptides; the 8-kDa domain (and also the intact 39-kDa pol β) requires prior incision of AP sites at their 5′ side for catalysis of β-elimination (*2*), while all other known AP lyases can carry out β-elimination at AP sites without preincision. Thus the 8-kDa domain is a good model system to elucidate the mechanisms of specific damage-recognition and β-elimination.

The structure of pol β has been determined by X-ray crystallography (*10*). The 8-kDa domain is connected to the rest of the enzyme (31-kDa domain) by a short linker peptide. It forms a stable globular fold with four α-helices arranged into a bundle (Figure 1A). α-Helices A and B are aligned antiparallel to each other, while α-helices C and D form a helix-hairpin-helix (HhH) motif, which is known as one of the common DNA binding motifs found in DNA repair enzymes (*11, 12*). The two sets of helices are packed against each other at an angle of approximately 30°.

Recently published studies provided important information to the understanding of these mechanisms. Piersen et al. (*13*) showed that pol β and its 8-kDa domain form a Schiff base intermediate with the dRP group in the process of

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[1] Abbreviations: pol β, DNA polymerase β; AP site, apurinic/apyrimidinic site; dRP, deoxyribose phosphate; HhH, helix-hairpin-helix; UDG, uracil-DNA glycosylase.

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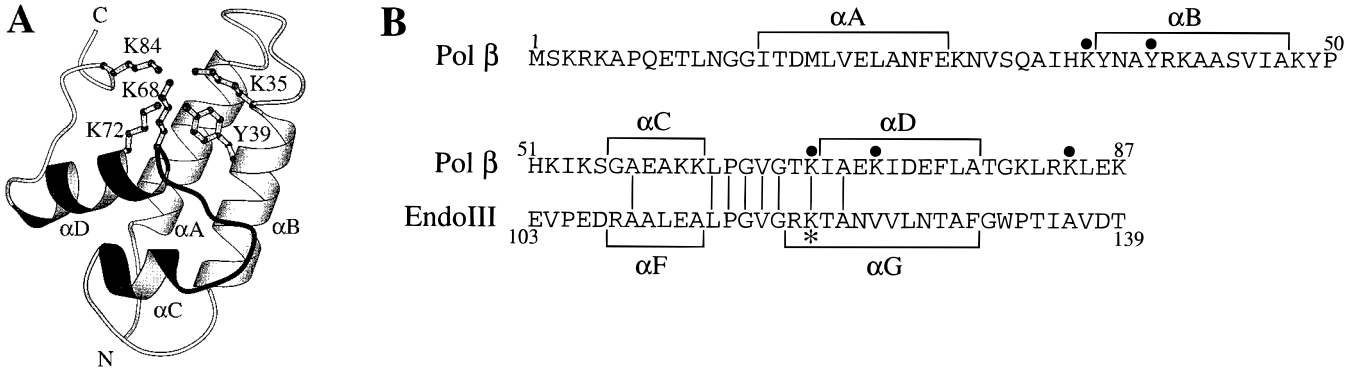


FIGURE 1: Structure of the amino-terminal 8-kDa domain of rat pol β. (A) Ribbon representation of the 8-kDa domain. Atomic coordinates were obtained from the Brookhaven Protein Data Bank (*23*) under accession code 2BPG. This figure was produced with MOLSCRIPT (*24*). Side chains of four lysine residues (amino acids 35, 68, 72, and 84) and a Tyr residue (amino acid 39) are also depicted. (B) Amino acid sequence of the 8-kDa domain. The amino acid sequence around the HhH motif of *E. coli* endonuclease III (endoIII) (*11*) is aligned. Four lysine and one tyrosine residues depicted in (A) are marked with dots. The lysine residue which is important for AP lyase activity of endonuclease III is marked with an asterisk. The regions forming α-helical structures are also indicated.

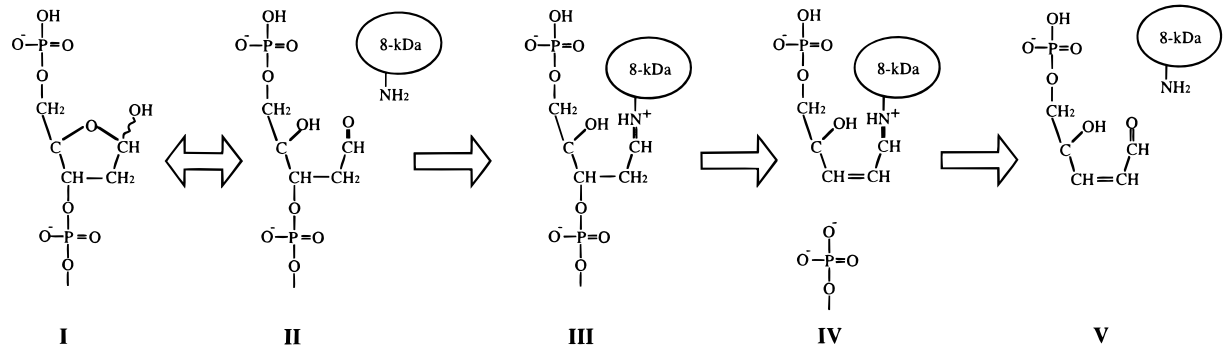


FIGURE 2: Reaction scheme for dRP excision catalyzed by the 8-kDa domain of pol β. I, a hemiacetal tautomer; II, a ring-opened aldehydic tautomer; III, a Schiff base intermediate; IV, a Schiff base intermediate after β-elimination of the 3′-phosphate; V, an unsaturated dRP group released from the 8-kDa domain.

β-elimination. This suggests that an amino group of either the amino terminus or one of the lysine residues of the 8-kDa domain may be directly involved in this reaction (Figure 2). Mullen and his colleagues (*14, 15*) proposed a model in which Lys-68 or Lys-72 could be the residue forming a Schiff base with the dRP group.

In the present study, we examined a series of site-directed mutations of the 8-kDa domain at positions which are likely to be essential for dRP excision. As a result, the catalytic center of pol β for the dRP excision was determined. We also investigated the effects of these mutations on damage-specific DNA binding and DNA polymerase activities. On the basis of these data, the molecular mechanism and structural features of pol β for dRP excision will be discussed.

EXPERIMENTAL PROCEDURES

Detection of Schiff Base Intermediates. The 21mer oligonucleotide (40 pmol) shown in Figure 3 was phosphorylated with [γ-^{32}P]ATP (4500 Ci/mmol) and T4 polynucleotide kinase. After inactivation of the kinase with 0.5% SDS and 12 mM EDTA, the labeled oligonucleotide was mixed with the 43mer and 20mer oligonucleotides (55 pmol each) shown in Figure 3, incubated at 70 °C for 5 min, and gradually cooled to 4 °C. The annealed oligonucleotides were precipitated in ethanol, dissolved in 50 μL of the buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM NaCl, and stored at 4 °C until use. More than 98% of the labeled oligonucleotide in the sample thus prepared was in a double-stranded form.

Reactions for Schiff base formation were conducted in the buffer containing 20 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, and 0.1 mg/mL acetylated gelatin. Indicated amounts of pol β or its derivatives, 0.25 unit of uracil-DNA glycosylase (UDG; purchased from Perkin-Elmer), and 2 μL of a freshly made solution of 0.5 M $NaBH_4$ were added to the reaction mixture whose final volume was 9 μL. In the reaction from which $NaBH_4$ was omitted, it was replaced with the same concentration of NaCl. The reaction was started by the addition of 1 μL of the annealed oligonucleotides and continued for 30 min at room temperature. The samples were then digested with 0.1 μg micrococcal nuclease in the presence of 10 mM $CaCl_2$ for 30 min at 37 °C. After addition of 10 μL SDS−PAGE loading buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 20 mM DTT, 10% glycerol, and 0.3 mg/mL bromophenol blue] and boiling for 1 min, the samples were loaded on a discontinuous polyacrylamide gel which consisted of 4% polyacrylamide in the running buffer (40 mM Tris-acetate, 2 mM EDTA, and 0.1% SDS) adjusted to pH 6.4 layered over 15% polyacrylamide in the running buffer (pH 7.4). Electrophoresis was performed in the running buffer (pH 7.4) at 10 V/cm for 2 h. The proteins in the polyacrylamide gel were transferred to an Immobilon-P^{SQ} membrane (purchased from Millipore) electrophoretically at 100 V for 1 h in the buffer containing 10 mM CAPS (pH 11) and 10% methanol. After drying up, the membrane was

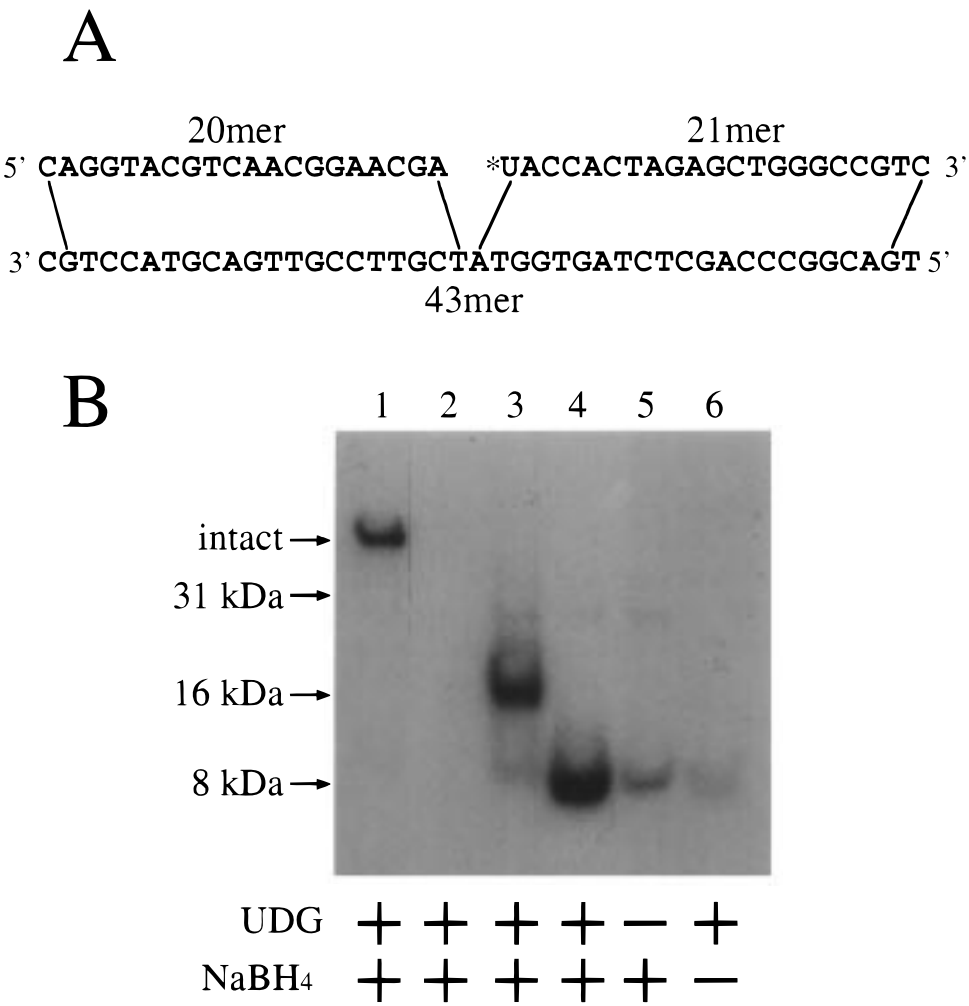


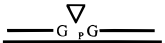
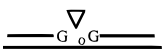
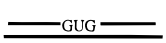
FIGURE 3: Formation of a Schiff base intermediate between pol β and a dRP group at a preincised AP site. (A) Structure of the DNA substrate for trapping a Schiff base intermediate. 20mer and 21mer upper-strand oligonucleotides were annealed to a 43mer lower-strand oligonucleotide. The 21mer oligonucleotide carrying a uracil residue was labeled with ^{32}P (designated by an asterisk) at its 5′ terminus before annealing. The two upper-strand oligonucleotides remained unsealed. (B) Covalent binding of a dRP group to pol β and its partial proteins. Schiff base intermediates were trapped with NaBH4 as described in Experimental Procedures. Fifty nanograms of either intact pol β (39 kDa; lane 1), the carboxyl-terminal 31-kDa (amino acids 88−355; lane 2), the amino-terminal 16-kDa (amino acids 1−151; lane 3), or the amino-terminal 8-kDa domain (amino acids 1−87; lanes 4 to 6) was incubated in the presence of uracil-DNA glycosylase (UDG) and $NaBH_4$ with the double-stranded oligonucleotide substrate shown in A. In lanes 5 and 6, UDG and $NaBH_4$ were omitted from the reaction, respectively. After incubation for 30 min at room temperature, the samples were digested with micrococcal nuclease and subjected to electrophoresis in an SDS-containing 15% polyacrylamide gel, transfer to a PVDF membrane, and autoradiography. The 31-kDa domain did not form a Schiff base intermediate because it does not have a dRP excision activity (*2*).

subjected to autoradiography with an X-ray film or a Fuji imaging plate for quantitative analysis.

Construction of Mutant Proteins. Site-directed mutations were introduced into the overexpression plasmid for the 8-kDa domain (*2*) by a PCR-based method (*16*) with the following primers: 5′-CGATCCACCGTTACAATGC-3′ and its complementary oligonucleotide for the K35R mutation, 5′-CGATCCACCAGTACAATGC-3′ and its complementary oligonucleotide for the K35Q mutation, 5′-TAGGAACACG-TATTGCTGA-3′ and its complementary oligonucleotide for the K68R mutation, 5′-TAGGAACACAGATTGCTGA-3′ and its complementary oligonucleotide for the K68Q mutation, 5′-TTGCTGAACGTATTGATGA-3′ and its complementary oligonucleotide for the K72R mutation, 5′-TTGCT-GAACAGATTGATGA-3′ and its complementary oligonucleotide for the K72Q mutation, 5′-AATTGCGTCGTCTG-GAAAA-3′ and its complementary oligonucleotide for the K84R mutation, 5′-AATTGCGTCAGCTGGAAAA-3′ and its complementary oligonucleotide for the K84Q mutation, and 5′-ACAATGCACAGAGAAAAGC-3′ and its complementary oligonucleotide for the Y39Q mutation. K72R and Y39Q mutations were also introduced into the overexpression plasmid for the intact 39-kDa pol β in a similar manner. Mutant 8-kDa domains and pol β were overexpressed from these plasmids and purified as previously described (*2*), except that the 8-kDa domain and pol β carrying the Y39Q mutation were overexpressed at 25 °C instead of 37 °C.

dRP Excision Assay. Excision of 5′-terminal dRP from preincised AP sites with the 8-kDa domain was measured as previously described (*2*). Briefly, the pBS−DNA (Stratagene) which carried a natural AP site and was labeled with ^{32}P at its *Eco*RI site (prepared as previously described in ref *3*) was digested with AP endonuclease, and subsequently incubated with indicated amounts of the 8-kDa domain for 30 min at 25 °C. After reducing the remaining 5′-terminal dRP with $NaBH_4$, the DNA was recovered, digested with *Hin*fI, and subjected to electrophoresis in a 20% polyacrylamide gel containing 8 M urea. Radioactivities of the DNA

Table 1: Affinity of the Wild-Type 8-kDa Domain to Various DNA Substrates

	DNA Substrate[a]	relative response[b]	K_d (nM) ± SD[c]
Oligo-I		1.6	200 ± 31
Oligo-II		0.97	370 ± 87
Oligo-III		2.0	55 ± 8
Oligo-IV		0.70	370 ± 43
Oligo-V		0.72	330 ± 100
Oligo-VI		0.39	850 ± 340
Oligo-VII		0.40	870 ± 300

[a] Arrows and triangles indicate a nick and a single-nucleotide gap, respectively. A synthetic AP site analogue, 5′-terminal phosphate, and hydroxyl groups are designated as F, P, and O, respectively. [b] Responses with 500 nM protein in surface plasmon resonance assays were normalized with the response with annealing of each upper strand oligonucleotide as shown in Figure 6C. [c] Dissociation constants were calculated as described in Experimental Procedures.

which still carried a dRP group and the DNA from which a dRP group was removed were measured with Fuji BAS1000.

DNA Binding Assay with a BIAcore System. All the oligonucleotides and proteins used in the binding assays with a BIAcore 1000 (BIAcore) were in buffer containing 20 mM HEPES-KOH (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, and 0.02% Triton X-100. A 5′-biotinylated oligonucleotide (5′-b-TCTACGGCCACACGTACCCTGCT-3′), which is the common lower-strand for oligo-I to -VII (Table 1), was immobilized on a streptavidin-conjugated Biosensor chip (BIAcore) by injecting 325 μL of the 0.5 μM oligonucleotide solution at 10 μL/min followed by 200 μL of 5 μM free biotin. This Biosensor chip carrying the lower strand oligonucleotide was used repeatedly through assay cycles consisting of the following four steps: a) injection of 15 μL of 2 μM upper-strand oligonucleotide at 2 μL/min; b) injection of 250 μL of various concentrations of the indicated 8-kDa domain at 30 μL/min followed by 100 μL of the buffer at the same flow rate; c) injection of 30 μL of 1 M KCl-containing buffer at 30 μL/min; d) injection of 30 μL of 30 mM NaOH at 30 μL/min. The upper-strand oligonucleotides used in these assays were 5′-GCAGGGTACG-3′ and 5′-phosphorylated FGTGGCCGTAG-3′ (F designates a 3-hydroxy-2-hydroxymethyltetrahydrofuran) for oligo-I; 5′-GCAGGGTACG-3′ and 5′-unphosphorylated FGTGGCCGTAG-3′ for oligo-II; 5′-GCAGGGTACG-3′ and 5′-phosphorylated GTGGCCGTAG-3′ for oligo-III; 5′-GCAGGGTACG-3′ and 5′-unphosphorylated GTGGCCGTAG-3′ for oligo-IV; 5′-GCAGGGTACG-3′ and 5′-phosphorylated TGTGGCCGTAG-3′ for oligo-V; 5′-GCAGGGTACGFGTGGCCGTAG-3′ for oligo-VI; 5′-GCAGGGTACGUGTGGCCGTAG-3′ for oligo-VII (see Table 1). Dissociation constants (K_d) were calculated from the equation, $1/R = (K_d/R_{max})(1/[8\text{-kDa domain}]) + 1/R_{max}$, in which R was the asymptote of the response to each concentration of the 8-kDa domain, and was obtained by extrapolation of the observed response curve (Figure 6A). Initial K_d and R_{max} values were obtained through regression of the double-reciprocal plot line (Figure 6B, inset), and subsequently the final values were determined by nonlinear least-squares regression analysis with the BMDP statistical package. The results shown in Tables 1 and 2 were obtained from the data with 50, 100, 200, 300, 400, and 500 nM of the 8-kDa domain.

Table 2: Affinities of Wild-Type and Mutant 8-kDa Domains to Damaged DNA[a]

8-kDa domain	relative response	K_d (nM) ± SD
wild-type	1.57	204 ± 31
K72R mutant	1.61	136 ± 17
K72Q mutant	1.47	179 ± 37
Y39Q mutant	0.94	475 ± 119

[a] Affinities to oligo-I (see Table 1) were calculated in the same manner as those of Table 1.

DNA Polymerase Assay. DNA polymerase activity of pol β was measured on a poly(dA)/oligo(dT) template/primer (molar ratio 5:1; total 0.5 μg) in 25 μL of the buffer containing 50 mM Tris-HCl (pH 8.8), 100 mM KCl, 0.5 mM $MnCl_2$, 0.4 mg/mL BSA, 1 mM DTT, and 50 μM [α-^{32}P]-TTP (5000−10000 cpm/pmol) with 1 ng of pol β at 25 °C. One unit of activity corresponds to the incorporation of 1 pmol of TMP into acid-precipitable material in 30 min.

AP Site Repair Assay. The repair reaction of natural AP sites was performed as previously described (*2*) with 14 ng of *Xenopus laevis* AP endonuclease and 0.2 unit of T4 DNA ligase (Gibco-BRL) on the circular DNA which was labeled with ^{32}P five nucleotides away on the 5′ side of a unique AP site. When indicated, 50 ng of pol β, either wild-type or one of the mutants, and/or 10 ng of the wild-type 8-kDa domain were added to the reaction.

RESULTS

Schiff Base Formation between pol β and a dRP Group. The β-elimination reaction on aldehydic AP sites proceeds through an abstraction of the 2′ proton (*17*). The substrate for this reaction is deduced to be either a free aldehyde or an iminium ion derived from a Schiff base formed between the carbonyl group of the aldehydic AP site and an amino group of the enzyme. Using a strong reducing agent, $NaBH_4$, Piersen et al. (*13*) was able to trap a cross-linking product between pol β and the AP site-containing DNA labeled with ^{32}P at the 3′ terminus, suggesting that β-elimination catalyzed by pol β may proceed via a Schiff base formation.

Applying the same techniques, we demonstrated that this Schiff base formation actually can be correlated to the dRP excision activity of pol β (Figure 3). In this experiment, we employed the intact pol β and three proteins which include the carboxyl-terminal 31-kDa domain (amino acids 88−335), the amino-terminal 16-kDa domain (amino acids 1−151), and the amino-terminal 8-kDa domain. These proteins were incubated with a double-stranded oligonucleotide containing a 5′-incised AP site whose 5′ phosphate was labeled with ^{32}P. After trapping the Schiff base intermediates by $NaBH_4$, we treated the samples with micrococcal nuclease to remove all the DNA except the ^{32}P-labeled dRP group from the cross-linking products. The ^{32}P-labeled products exhibited the electrophoretic mobilities indistinguishable

from those of the respective unreacted proteins except for the 31-kDa domain, which did not form any cross-linking product with the ^{32}P-labeled dRP (Figure 3B, lane 2). This observation is consistent with our previous biochemical data in which the 31-kDa domain failed to excise dRP groups from AP sites (*2*). The cross-linked products were hardly detected when either uracil-DNA glycosylase (UDG), which converts a uridine to an AP site, or $NaBH_4$ was omitted from the reaction (Figure 3B, lanes 5 and 6). When nuclease treatment was omitted from the assay, another radioactive band which migrated more slowly than each protein was detected in addition to the band migrating with each unreacted protein (data not shown). These products with slower mobilities were those which remained covalently bound to the oligonucleotide. A similar species of products was observed by Piersen et al. (*13*). This indicates that Schiff bases can be trapped at either before or after β-elimination releases dRP from the rest of the DNA.

Effects of Site-Directed Mutations on dRP Excision. To identify which specific residues on the 8-kDa domain forms Schiff base with dRP, we carried out a number of knowledge-based site-directed mutagenesis on the lysine residues. *Escherichia coli* endonuclease III is the protein in which an HhH motif was originally identified (*11*). Its HhH motif contains an active center for β-elimination. Mutation at Lys-120, which is located at the mouth of a catalytic pocket in the HhH motif, completely abolishes the activity of the enzyme. Based on the structural similarity between the HhH motifs of endonuclease III and the 8-kDa domain of pol β, it was proposed that Lys-68 of the 8-kDa domain, which corresponds to Lys-120 of endonuclease III, is the catalytic center (*14*; Figure 1B). However, after careful examination of the structures of both pol β and endonuclease III, a profound difference was identified. Although the 8-kDa domain of pol β contains the familiar HhH DNA binding motif (αC, loop, and αD in Figure 1A), it lacks the apparent catalytic pocket found in endonuclease III. Instead, the 8-kDa domain has a distinctive basic patch which is formed by Lys-35, Lys-68, Lys-72, and Lys-84. This basic patch may serve as a DNA binding surface for subsequent catalytic activity. In addition, Tyr-39 is also in close proximity to the basic patch. Since an aromatic residue is critical for β-elimination by tripeptide AP lyases (*9*), Tyr-39 of pol β may play a similar role for the dRP excision.

To evaluate the involvement of the lysine and tyrosine residues in the dRP excision reaction, we introduced point mutations at these positions and measured the excision activity of the mutated 8-kDa domains. Arg and Gln were chosen as substitutes for Lys in order to minimize possible structural alterations. Gln was substituted for Tyr to replace an aromatic residue while keeping a possible hydrogen bonding with one of the lysine residues. As a result, Lys-72→Arg (K72R), Lys-72→Gln(K72Q) and Tyr-39→Gln-(Y39Q) completely lost the dRP excision activity, while the activities of 8-kDa domains with mutations at either Lys-35, Lys-68, or Lys-84 were comparable to that of the wild-type 8-kDa domain (Figure 4).

The three mutants with no dRP excision activity were further examined for their ability to form Schiff base intermediates. The amount of cross-linked products formed between dRP groups and the mutant 8-kDa proteins were less than 5% of those formed with the wild-type 8-kDa

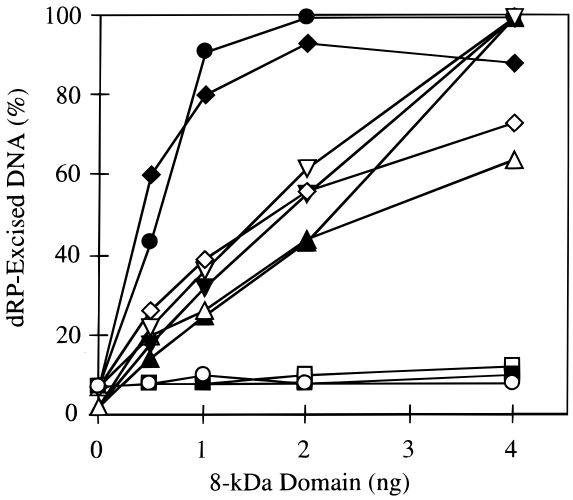


FIGURE 4: Excision of dRP groups by mutant 8-kDa domains. Excision activity was measured as previously described (*2*) for the following proteins: the wild-type 8-kDa domain (●), K35R mutant 8-kDa domain (▲), K35Q mutant 8-kDa domain (△), K68R mutant 8-kDa domain (▼), K68Q mutant 8-kDa domain (▽), K72R mutant 8-kDa domain (■), K72Q mutant 8-kDa domain (□), K84R mutant 8-kDa domain (◆), K84Q mutant 8-kDa domain (◇), and Y39Q mutant 8-kDa domain (○).

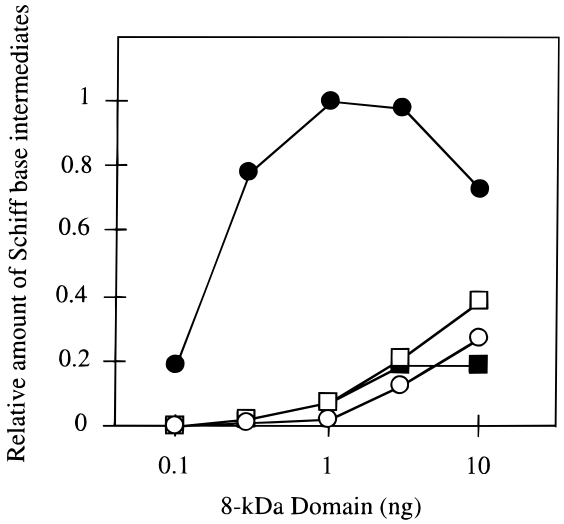


FIGURE 5: Effect of mutations of the 8-kDa domain on covalent binding to the dRP group. Schiff base intermediates were formed on indicated amounts of either the wild-type 8-kDa domain (●), K72R mutant 8-kDa domain (■), K72Q mutant 8-kDa domain (□) or Y39Q mutant 8-kDa domain (○), and trapped with $NaBH_4$ and analyzed as described in Experimental Procedures, and as in Figure 3. The radioactivity of dRP-bound proteins was measured with the Fuji BAS1000 and normalized with the activity of 1 ng of the wild-type 8-kDa protein as a standard.

protein in the presence of 1 ng of the protein (Figure 5). As more 8-kDa protein was added to the reaction, however, the Schiff bases formed with the mutant proteins started to increase, while those with the wild-type protein did not increase. It is likely that, when added in an excess amount, the mutant 8-kDa proteins which have at least 15 amino groups might be able to carry out β-elimination with one of the remaining 15 (16 in the case of Y39Q) amino groups at a lower rate as it is the case with basic proteins such as histones. Thus, a strong correlation between the decrease of Schiff base formation and the loss of dRP excision activity is observed in the three mutants, supporting the model in which β-elimination proceeds through Schiff base formation.

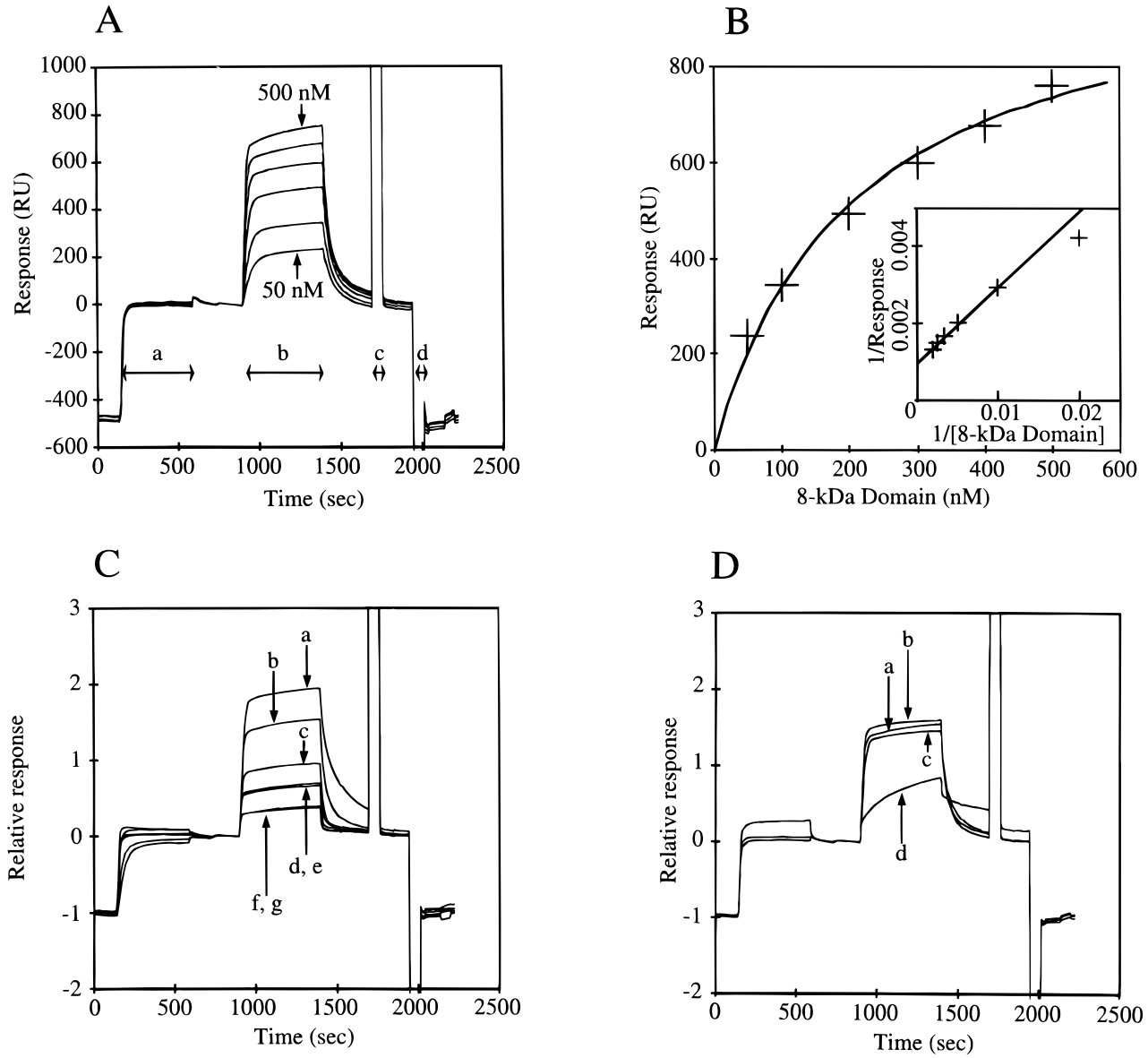


FIGURE 6: Specific binding of the 8-kDa domain to damaged DNA. (A) Binding of the wild type 8-kDa domain to the double-stranded oligonucleotide carrying a 5′-incised synthetic AP site analogue (oligo-I, see Table 1). Association and dissociation of the protein to the immobilized oligonucleotide were analyzed by measuring surface plasmon resonance with BIAcore 1000 (BIAcore), as described in Experimental Procedures: a, annealing of the upper strand oligonucleotide; b, loading of the 8-kDa domain (50, 100, 200, 300, 400, and 500 nM); c, washing with 1 M KCl-containing buffer; d, washing with 30 mM NaOH for regeneration of single-stranded DNA. (B) Plot of the response against the concentration of the 8-kDa domain. The asymptote of response for each concentration of the protein was obtained from the experiment shown in A as described in Experimental Procedures. A regression line was obtained through double-reciprocal plotting (inset) and subsequent statistical fitting. (C) Binding of the wild-type 8-kDa domain to various double-stranded oligonucleotides. Association and dissociation of the protein were analyzed in the same manner as for (A). The oligonucleotides used are shown in Table 1: a, oligo-III; b, oligo-I; c, oligo-II; d, oligo-V; e, oligo-IV; f, oligo-VII; g, oligo-VI. (D) Binding of the wild-type and mutant 8-kDa proteins to the double-stranded oligonucleotide carrying a 5′-incised synthetic AP site analogue (oligo-I, see Table 1). Association and dissociation of the proteins were analyzed in the same manner as for (A). The proteins used were wild-type (a), K72R (b), K72Q (c), and Y39Q mutant (d).

Damage-Specific Binding of the 8-kDa Domain. Since the 8-kDa domain has a specific activity of dRP excision which is much higher than that of the histones and polyamines (*2*), it is likely that the 8-kDa domain may specifically recognize and bind to damaged DNA with AP sites. The 8-kDa domain is reported to have high affinity to single-stranded DNA but to exhibit limited affinity to double-stranded DNA (*18*). The affinity of the 8-kDa domain to damaged DNA has not been examined. To analyze this possible damage-specific DNA binding, we employed a surface plasmon resonance biosensor (BIAcore) with a series of double-stranded oligonucleotides with various damaged structures shown in Table 1. In these assays, it is not possible to use DNA with a preincised natural AP site since it could be quickly converted to a single-nucleotide gapped DNA by the 8-kDa domain. We designed a DNA oligomer with a nicked 3-hydroxy-2-hydroxymethyltetrahydrofuran (tetrahydrofuran) as a substitute, where the synthetic analogue of the AP site cannot be excised by the 8-kDa domain via β-elimination (*2*). Figure 6A shows the data of a typical experiment, in which the interaction of the 8-kDa protein at indicated concentrations with the immobilized

Table 3: DNA Polymerase Activity of Wild-Type and Mutant pol β

pol β	wild-type	K72R	Y39Q
specific activity (unit/ng)[a]	12.7 ± 1.9	14.3 ± 1.5	0 ± 0.1

[a] Specific activity was measured as described in Experimental Procedures. Averages ± standard deviations were calculated from three assays.

oligonucleotide was measured by a refractive index change in the BIAcore 1000 system. Since the dissocated 8-kDa domain molecules appeared to re-associate with the immobilized DNA at a significantly high rate under these experimental conditions, it was difficult to otain its association rate and dissociation rate as independent values. Therefore, we calculated the dissociation constant (K_d) in a equilibrated state as described in Experimental Procedures (see also Figure 6B).

Among the tested oligonucleotides, the DNA carrying a single-nucleotide gap with a 5′-phosphate group (oligo-III) had the highest affinity to the 8-kDa domain, followed by the DNA carrying a nicked synthetic AP site with a 5′-phosphate group (oligo-I) (Figure 6C and Table 1). DNA oligomers without the 5′-phosphate group at the strand-break site (oligo-II and oligo-IV) showed strongly diminished affinity to the 8-kDa domain. This result is consistent with the observation by Prasad et al. (*19*) that binding of pol β to a gapped DNA is strongly enhanced by the presence of a 5′-phosphate group on the downstream oligonucleotide. The 8-kDa domain did not bind strongly to DNA substrates with intact phosphate backbones (oligo-VI and oligo-VII). The DNA nicked at a thymidine site (oligo-V) was also a poor substrate for binding. The three 8-kDa mutants, K72R, K72Q, and Y39Q, were also examined for their binding activity to the DNA oligomer with a nicked tetrahydrofuran group. The K72R and K72Q mutants showed high affinity to the damaged DNA comparable to that of the wild-type 8-kDa domain, whereas Y39Q mutant exhibited a low binding activity (Table 2). Furthermore, the response curve of BIAcore indicated a distinct pattern with Y39Q (Figure 6D), suggesting that the association rate and dissociation rate of this mutant to the damaged DNA could be much slower than those of the wild-type.

DNA Synthesis and Repair by Mutant pol β. The data presented so far indicate that the Lys-72 and Tyr-39 residues are critical for dRP excision by pol β. To test whether these residues are also involved in the DNA synthesis, we introduced K72R and Y39Q mutations to the intact pol β. Both mutant and wild-type enzymes were assayed for DNA polymerase activity on poly(dA):oligo(dT) templates (Table 3). Pol β-K72R exhibited a polymerase activity similar to that of the wild-type enzyme. On the other hand, less than 1% of the polymerase activity remained with pol β-Y39Q.

These two pol β mutants were further examined for AP site repair. As previously reported (*2*), in the presence of AP endonuclease and DNA ligase, the wild-type pol β was able to repair AP sites in a highly efficient manner. As shown in Figure 7 (lanes 5 and 7), the K72R or Y39Q mutant pol β failed to repair AP sites. Since we used the DNA prelabeled at the site five nucleotides away from the AP site toward 5′, these assays allowed us to detect intermediate products which were elongated by a few nucleotides by DNA synthesis. The products from pol β-K72R indeed became

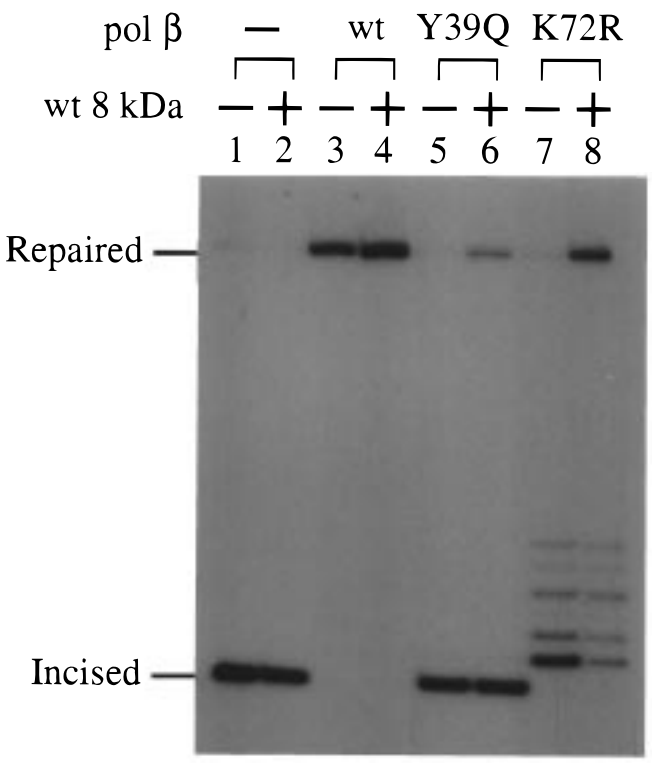


FIGURE 7: AP site repair with mutant pol β. Using an AP site-containing circular DNA which was prelabeled with ^{32}P at a distance of five nucleotides from the AP site toward 5′, the repair reactions were conducted as previously described (*2*) with *X. laevis* AP endonuclease, bacteriophage T4 DNA ligase, and the following proteins: no intact pol β (lanes 1 and 2), 50 ng of the wild-type rat pol β (lanes 3 and 4), 50 ng of the pol β with a Y39Q mutation (lanes 5 and 6), and 50 ng of the pol β with a K72R mutation (lanes 7 and 8). Ten nanograms of the wild type 8-kDa domain was also added to the repair reactions for lanes 2, 4, 6, and 8. After incubation at 25 °C for 30 min, the DNA samples were recovered, digested with *Hin*fI, and analyzed by electrophoresis through an 8 M urea-containing 20% polyacrylamide gel.

longer by up to six nucleotides. In contrast, the intermediate products from the reaction with pol β-Y39Q showed no change in size of the DNA incised by AP endonuclease. This difference may result from DNA polymerase activities remaining in the two mutant enzymes as shown above. Interestingly, the length of six nucleotides which pol β-K72R added to the incised DNA is in agreement with the number of nucleotides which pol β can synthesize in a processive manner (*20*).

The inability of pol β-K72R to repair AP sites may be a consequence of its loss of dRP excision activity. To examine whether a separate dRP lyase activity is able to complement the mutant pol β for AP site repair, we added the wild-type 8-kDa protein to the repair reaction. In the reactions with or without the wild-type pol β (Figure 7, lanes 2 and 4), the additional 8-kDa protein did not have any effect on the AP site repair. In contrast, the repair with pol β-K72R was restored up to 49% by the addition of the 8-kDa domain (Figure 7, lane 8). It is noticeable that an intermediate product which resulted from addition of one nucleotide to the incised DNA was decreased as the repair was complemented, while the longer intermediate products remained at the same level. Such longer products could not be successfully repaired since the reconstituted system used here did not include any nuclease which could remove extra nucle-

otides prior to ligation. This observation suggests that a coordinated action of dRP excision and DNA synthesis could be important for efficient repair of the AP site by pol β. The repair with pol β-Y39Q was also restored, but to a lesser extent (Figure 7, lane 6).

DISCUSSION

Mutational analysis of the 8-kDa domain derived from the amino-terminus of pol β indicated that Lys-72 is an essential residue for the β-elimination reaction. It is responsible for dRP excision from the 5′-incised AP sites by forming a Schiff base with dRP. Mutations on residues, Lys-35, Lys-68, and Lys-84, did not eliminate the dRP excision activity, although they along with Lys-72 constitute the basic patch on the 8-kDa domain. These results strongly suggest that Lys-72 may be the site of transimination. Piersen et al. (*13*) reported that the K72A mutant of pol β could be trapped as a Schiff base intermediate, albeit at the 30% efficiency of that of the wild-type pol β, suggesting possible involvement of a residue other than Lys-72 as the transimination site. However, the amounts of pol β used in their trapping experiments were in great excess. It is likely that an amino group from other lysine residues of the lysine-rich 8-kDa domain may contribute to the Schiff base formation with an AP site at an apparent lower rate even when the major site, Lys-72, is mutated. The result shown in Figure 5 supports this possibility.

The results supporting Lys-72 as the catalytic center provide a solid foundation for establishing a reaction mechanism for dRP excision by pol β. By inferring from the results of mutational data on endonuclease III, where the substitution of Lys-120 by Gln resulted in complete elimination of its catalytic activity (*11*), Lys-68 of pol β was suggested to be the catalytic center of dRP excision. Mullen and Wilson (*14*) proposed a model for the mechanism of detection and excision of an AP site by the 8-kDa domain of pol β on the basis of the three-dimensional structure of the 8-kDa domain determined by NMR (*21*) and its structural and functional similarities to endonuclease III. This model suggests Lys-68 as the residue to form a Schiff base, while His-34 assists displacement of the 1′-OH group and deprotonation of C2′. Lys-35 is proposed to play a role in stabilization and protonation of the leaving group, the phosphate on the C3′. Later, the model was modified to include Lys-72 as a part of the catalytic mechanism (*15*). However, little experimental evidence was presented to support their model. One potential weakness of this model is that it heavily relies on the assumption that endonuclease III and the 8-kDa domain of pol β may excise dRP by a similar enzymatic mechanism. Endonuclease III has a well-defined catalytic pocket where only one lysine residue, Lys-120, lies. In contrast, pol β carries a basic patch consisting of four lysine residues near the catalytic center. Therefore, although an HhH motif is found in the catalytic domain of both endonuclease III and the 8-kDa domain, their modes of interaction to damaged DNA may be distinct from each other. Endonuclease III has DNA-*N*-glycosylase activity and is suggested to form a Schiff base intermediate with urea- and thymine glycol-containing DNA at higher rates than with AP site-containing DNA (*22*). In addition, endonuclease III can incise intact AP sites, whereas incision of AP sites at the 5′ side by AP endonuclease is a prerequisite to β-elimination by pol β (*2*). These characteristics also suggest that endonuclease III and pol β interact with damaged DNA in different manners.

The results obtained with the Y39Q mutation indicate that Tyr-39 might be a critical residue for the function of the 8-kDa domain as a dRP lyase. A model has been proposed for the tripeptide AP lyase activity, in which the aromatic ring of either tryptophan or tyrosine residue can recognize the AP site through stacking interactions (*8*, *9*). Tyr-39 in the 8-kDa domain may serve for AP site recognition in a similar manner. This possibility is supported by the result that Y39Q exhibited a relatively low affinity to the damaged DNA compared to that of the wild-type 8-kDa domain. Interestingly, conversion of Tyr-39 to Gln abolished the DNA polymerase activity of pol β. In addition, we observed that the 8-kDa Y39Q protein became insoluble when overproduced in bacteria at 37 °C, whereas the wild-type 8-kDa and other mutant proteins were in a soluble fraction in the same condition (data not shown). This observation suggests that Tyr-39 may be important for the proper folding of the 8-kDa domain. Deciphering the precise function of Tyr-39 awaits further investigations.

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