

Mutations in the $\alpha 8$ Loop of Human APE1 Alter Binding and Cleavage of DNA Containing an Abasic Site*

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Jiang-Cheng Shen[‡] and Lawrence A. Loeb[§]

From the Department of Pathology, University of Washington, Seattle, Washington 98195-7705

Recent crystallographic studies reveal loops in human AP endonuclease 1 (APE1) that interact with the major and minor grooves of DNA containing apurinic/apyrimidinic (AP) sites. These loops are postulated to stabilize the DNA helix and the flipped out AP residue. The loop $\alpha 8$ interacts with the major groove on the 3' side of the AP site. To determine the essentiality of the amino acids that constitute the $\alpha 8$ loop, we created a mutant library containing random nucleotides at codons 222–229 that, in wild-type APE1, specify the sequence NPKGNNKK. Upon expression of the library (2×10^6 different clones) in *Escherichia coli* and multiple rounds of selection with the alkylating agent methylmethane sulfonate (MMS), we obtained $\sim 2 \times 10^5$ active mutants that complemented the MMS sensitivity of AP endonuclease-deficient *E. coli*. DNA sequencing showed that active mutants tolerated amino acid substitutions at all eight randomized positions. Basic and uncharged polar amino acids together comprised the majority of substitutions, reflecting the positively charged, polar character of the wild-type loop. Asn-222, Asn-226, and Asn-229 exhibited the least mutability, consistent with x-ray data showing that each asparagine contacts a DNA phosphate. Substitutions at residues 226–229, located nearer to the AP site, that reduced basicity or hydrogen bonding potential, increased K_m 2- to 6-fold and decreased AP site binding; substitutions at residues 222–225 exhibited lesser effects. This initial mutational analysis of the $\alpha 8$ loop supports and extends the conclusion of crystallographic studies that the loop is important for binding of AP-DNA and AP site incision.

Human apurinic/apyrimidinic endonuclease 1 (APE1)¹ is a multifunctional enzyme that possesses at least two distinct biological functions within the same polypeptide, *i.e.* DNA repair and protein reduction-oxidation (redox) (1). The C-terminal segment encodes an apurinic/apyrimidinic (AP) endonuclease activity that plays a pivotal role in base excision repair (BER), and the N-terminal segment is responsible for the redox

activity. Structural analysis of APE1 bound to abasic DNA has revealed loop domains that interact with the helical grooves in DNA (2). APE1 is postulated to stabilize the flipped out abasic residue by a double-loop mechanism involving interactions with both the minor and the major grooves at the AP site (2). Other loop domains also interact with DNA on either the 5' or the 3' side of the AP residue to facilitate the formation of a stable APE1-DNA complex (2). These loop domain interactions may therefore play an important role in governing AP endonuclease activity, particularly with respect to the DNA substrate specificity.

In addition to AP endonuclease activity that cleaves DNA 5' of the AP site, human APE1 encodes a 3'-exonuclease activity that removes nucleotides from the 3' terminus at nicks, gaps, and 3'-recessed breaks in DNA (3). Recently, this activity has been shown to preferentially remove 3' mismatches *in vitro* and thus may serve as a proofreading exonuclease for polymerase β in BER (4). APE1 also possesses phosphatase and phosphodiesterase activities that remove, respectively, 3'-phosphate and 3'-blocking groups, such as phosphoglycolates (5), β -L-dioxolane-cytidine (6), azidothymidine (4), and 3'-terminal residues resulting from glycosylase/lyase reactions (7). These activities that excise altered nucleotides at DNA breaks might thus facilitate critical repair processes in cells, such as ligation and repair synthesis (8, 17). Notably, these 3'-end-processing activities encoded by human APE1 are mediated at the same active site as the robust AP endonuclease activity. Although these 3'-processing activities are detectable *in vitro*, they are much weaker (>100 -fold) than the AP endonuclease activity. In contrast, in the case of *Escherichia coli* exonuclease III (ExoIII), the bacterial counterpart of APE1, the AP endonuclease and 3'-processing activities are comparable (9), suggesting that human APE1 might have evolved to be more stringent in selection of DNA substrates.

The DNA-interacting loops of *E. coli* ExoIII and human APE1 occupy similar locations in crystal structures, and most are superimposable (10). Of the loop domains in ExoIII, loop αM , which corresponds structurally to loop $\alpha 8$ in human APE1, is of particular interest (11). Insertion of this loop domain into calf DNase I endows this enzyme with AP endonuclease activity, suggesting a critical role for loop αM in AP site recognition and binding. In APE1-DNA complexes, loop $\alpha 8$ (amino acids 222–229: NPKGNNKK) is in close contact with the major groove 3' of the abasic residue (2). A chain of three asparagine residues (Asn-222, Asn-226, and Asn-229) lines up along the major groove and contacts two 3' AP-DNA phosphates. These interactions are believed to stabilize the distorted DNA structure that bears an extra-helical baseless residue and may also facilitate AP site recognition and binding.

To assess the essentiality of amino acid residues in the $\alpha 8$ loop, and to gain insight into their function in DNA binding and catalysis, we constructed a mutant library containing random

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[‡] Present address: Laboratory of Human Carcinogenesis, NCI, National Institutes of Health, Bethesda, MD 20892-4255.

[§] To whom correspondence should be addressed: Dept. of Pathology, Box 357705, University of Washington, HSB, K-072, 1959 NE Pacific Ave., Seattle, WA 98195-7705. Tel.: 206-543-6015; Fax: 206-543-3967; E-mail: laloeb@u.washington.edu.

¹ The abbreviations used are: APE1, AP endonuclease 1; AP, apurinic/apyrimidinic; BER, base excision repair; LB, Luria-Bertani; MMS, methylmethane sulfonate; PBS, phosphate-buffered saline; ExoIII, exonuclease III; DTT, dithiothreitol; nt, nucleotide(s).

nucleotides in place of the eight wild-type codons specifying the $\alpha 8$ loop. We used functional complementation of AP endonuclease-deficient *E. coli* to select for active APE1 mutants that conferred resistance to the alkylating agent MMS. Despite evolutionary conservation of the $\alpha 8$ loop, we observed that all eight amino acids are substitutable. Together, basic and uncharged polar amino acids constituted the majority of replacements, in keeping with the positively charged, polar nature of the loop. The substitutions can alter binding of an AP-site-containing oligonucleotide and AP endonuclease activity, suggesting a critical role for the $\alpha 8$ loop in modulation of AP-DNA binding and catalytic activities. Our analysis of 187 active mutants constitutes an extensive examination of the effects of mutation in the $\alpha 8$ loop on the function of APE1 *in vivo* and *in vitro*.

EXPERIMENTAL PROCEDURES

Construction of Human APE1 Random Mutant Library—The APE1 gene was isolated from a Quick-clone human HeLa cDNA library (BD Biosciences, Palo Alto, CA) by PCR amplification using the flanking oligonucleotides 5'-ATGCCGAAGCGTGGGAAAAAGGGA-3' and 5'-CAGTGCTAGGTATAGGGTGATAGG-3' (Qiagen, Alameda, CA) as primers. The PCR-amplified DNA fragment was gel-purified and cloned into the TOPO TA-cloning vector pTrcHis2-TOPO (Invitrogen, Carlsbad, CA); the resulting plasmid was designated pTrcHis2-APE1. The orientation and nucleotide sequence of the cloned human APE1 gene were confirmed by using an ABI Prism DNA sequencer.

The APE1 library containing random nucleotides in place of the eight wild-type codons specifying the $\alpha 8$ loop was constructed by a two-step PCR amplification methodology. First, two DNA fragments encoding the N-terminal and C-terminal portions of the APE1 cDNA with a 39-nucleotide overlapping sequence were PCR-amplified separately. The N-terminal fragment was amplified by using primers 5'-ATGCCGAAGCGTGGGAAAAAGGGA-3' and 5'-GCGAAGGTCAATTTCTTCA-TGTGCCACATTGAGGTCTCC-3' (Qiagen). For the C-terminal fragment with random mutations, the forward primer 5'-GGAGACCTCA-ATGTGGCACATGAAGAAATTGACCTTCGCAACCCCAAGGGGAAC-AAAAAGAATGCTGGCTTCACGCCACAAGAGCGCC-3' (Integrated DNA Technologies, Coralville, IA) and the reverse primer 5'-CAGTGCTAGGTATAGGGTGATAGG-3' (Qiagen) were used for PCR amplification. The underlined letters indicate the loop $\alpha 8$ region that was synthesized using a mixture containing 70% of the canonic nucleotides and 10% of each of the other three nucleotides at each position. The two fragments were gel-purified, then were used in the second step of PCR amplification. Because of a 39-nucleotide terminal sequence overlap, PCR amplification with these fragments can produce a single DNA fragment that integrates both sequences. Using a reaction mixture containing both fragments and a primer set of the forward primer used for the N-terminal fragment 5'-ATGCCGAAGCGTGGGAAAAAGGGA-3' and the reverse primer used for the C-terminal fragment 5'-CAGTGCTAGGTATAGGGTGATAGG-3' in the PCR, we obtained a DNA product that spans both fragments and contains random mutations in the loop $\alpha 8$ sequence.

The composite DNA fragment was gel-purified and then digested with restriction enzymes SacI, which cleaves near the 5' terminus, and PstI, which cleaves near the 3' terminus. The digested SacI-PstI fragment was gel-purified and ligated into a SacI-PstI pTrcHis2-APE1 vector. The vector was produced by digestion of a "dummy" pTrcHis2-APE1 plasmid with both restriction enzymes. The dummy APE1 plasmid contains an altered amino acid sequence in the loop $\alpha 8$ (IPRANKN in contrast to the wild-type NPKGNKKK) and has been demonstrated to confer little or no resistance to MMS in AP endonuclease-deficient *E. coli* BW542 (see Fig. 4, B and C). The use of a dummy plasmid instead of the wild-type APE1 plasmid in the cloning process mitigates against selection of any contaminating wild-type APE1 in the library. After ligation, plasmids containing the random library were used to transform *E. coli* Top10 cells, and the number of transformants was determined on LB/agar plates containing 100 μ g/ml ampicillin to obtain the library size. After transformation, solutions were incubated with Luria-Bertani (LB) broth (1:30) in the presence of 100 μ g/ml ampicillin at 37 °C overnight, and plasmids were then isolated by using a Qiagen maxiprep kit.

Genetic Selection for Active APE1 Mutants—*E. coli* BW542 (*xth-9, nfo::kan*), a generous gift from Dr. Bernard Weiss, University of Michigan, Ann Arbor, was transformed with plasmids containing the APE1

random library (diversity: 2×10^6), and the transformed cultures (1.8×10^7 cells in 11 ml) were grown overnight at 37 °C in 300 ml of LB broth containing 100 μ g/ml ampicillin and 50 μ g/ml kanamycin. This mutant APE1/BW542 library was then subjected to sequential rounds of MMS exposure and selection. First, the cell culture in stationary phase was inoculated at a 1/10 ratio in LB broth containing necessary antibiotics and incubated at 37 °C in a shaker at 225 rpm for 90 min. The proliferated cell culture was precipitated by centrifugation, and cell pellets were re-suspended in 1 \times phosphate-buffered saline (PBS, Invitrogen). Next, a 30-min starvation was carried out by incubation of the cells in 1 \times PBS at 37 °C in a shaker at 225 rpm. After starvation, MMS (0.5 μ l/ml) (Sigma-Aldrich, St. Louis, MO) was added to the culture in 1 \times PBS, and the culture was incubated in a 225-rpm shaker at 37 °C for 15 min. Following MMS treatment, cells were harvested by centrifugation and pellets were re-suspended and washed with 1 \times PBS. After 1 \times PBS wash, cells were centrifuged again and pellets were re-suspended in LB broth. Finally, the re-suspended cells in LB broth were inoculated 1/10 in LB broth containing antibiotics, and cells were grown overnight at 37 °C in a 225-rpm shaker to obtain cultures for the next round of MMS selection. In parallel, we also determined the survival ratio of the mutant library comparing to the wild-type APE1/BW542 and the parental strain BW35 after each round of MMS treatment by counting surviving colonies on LB-agar plates containing necessary antibiotics. This allowed us to monitor the progress of selection and to record the size of the resistant library after each round of MMS treatment. In this study, the random library underwent eight rounds of selection with slightly increasing concentrations of MMS (round 1 at 0.5 μ l/ml, round 2 at 1.0 μ l/ml, and rounds 3–8 at 2.0 μ l/ml). To avoid accumulation of mutations in the host cells from MMS exposure, we isolated the plasmids after round 5 and re-transformed BW542 for the subsequent rounds of selection.

MMS Gradient Plates—LB-agar plates containing linear concentration gradient of MMS were prepared following the method by Cunningham *et al.* (12). Briefly, 25 ml of molten LB-agar (50 °C) containing a designated concentration of MMS was poured in a 9-cm square polystyrene Petri dish, which was tilted with an angle allowing zero thickness of the LB-agar at the lifted edge. After the agar solidified, the plate was leveled and 25 ml of molten LB-agar without MMS was poured in the plate to overlay the MMS-containing agar. A linear MMS concentration gradient thereby forms in the agar plate from zero to the designated concentration of MMS along the tilted direction. Notably, 25 μ l of 10% Antifoam B Emulsion (Sigma-Aldrich) was added to each 25-ml LB-agar molten solution to prevent meniscus formation in the Petri dish.

Bacterial cultures to be tested on the gradient plates were grown to the stationary phase, and 20 μ l of the culture was mixed with 2 ml of molten soft agar (7.5%; 45 °C). While applying bacteria on the gradient plate, a microscope slide (Fisher Scientific, Pittsburgh, PA) was employed by using its 7.5-cm edge to dip in the bacteria-soft agar solution and then lightly touch the surface of the plate parallel to the MMS gradient. Plates were incubated at 37 °C overnight (18–20 h), and cell growth could then be visualized. For photography, 1.5 ml of an acridine orange solution (0.2 mg/ml in ethanol; Sigma-Aldrich) was added to the surface of the plate, and photographs were taken under UV irradiation.

Expression and Purification of Human APE1 Protein—The pTrcHis2-TOPO cloning vector used to clone the open reading frame of the human APE1 gene is also an *E. coli* expression vector. Expression of the wild-type as well as various mutant APE1 proteins is driven and regulated by a cassette of *trc* promoter/*lacO* operator and a plasmid encoded *lac* suppressor gene, *lacI^q* (Invitrogen, Carlsbad, CA). The encoded gene in this vector will fuse with an *myc* epitope and a six-histidine tag at the C terminus rendering the expressed protein convenient for purification. We expressed wild-type and several mutant APE1 proteins in *E. coli* Top10 cells by growing 1% inoculation cultures (in LB broth with 100 μ g/ml ampicillin) at 37 °C for about 3 h to mid-log phase ($A_{600} = 0.5$ – 0.6). Thereafter, 1 mM isopropyl-1-thio- β -D-galactopyranoside was added to the cultures for induction of gene overexpression, and incubation was continued for an additional 2 h. Cells were harvested by centrifugation and were re-suspended at $\sim 10^{10}$ cells/ml in 1 \times binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) (His-Bind Kits, Novagen, Madison, WI) containing 0.25 mg/ml lysozyme and 1 mM phenylmethylsulfonyl fluoride. To obtain cell extracts, cell suspensions were first frozen at -80 °C and then thawed on ice for 2 h. Soluble extracts were obtained by centrifugation and applied to the His-Bind Ni²⁺-affinity column, and proteins were purified according to the manufacturer's protocol (Novagen). Fractions containing eluted protein solutions were dialyzed and stored in buffer containing 10% glycerol, 125 mM NaCl, 20 mM Tris-HCl, pH 8.0, and 1 mM DTT. Purified

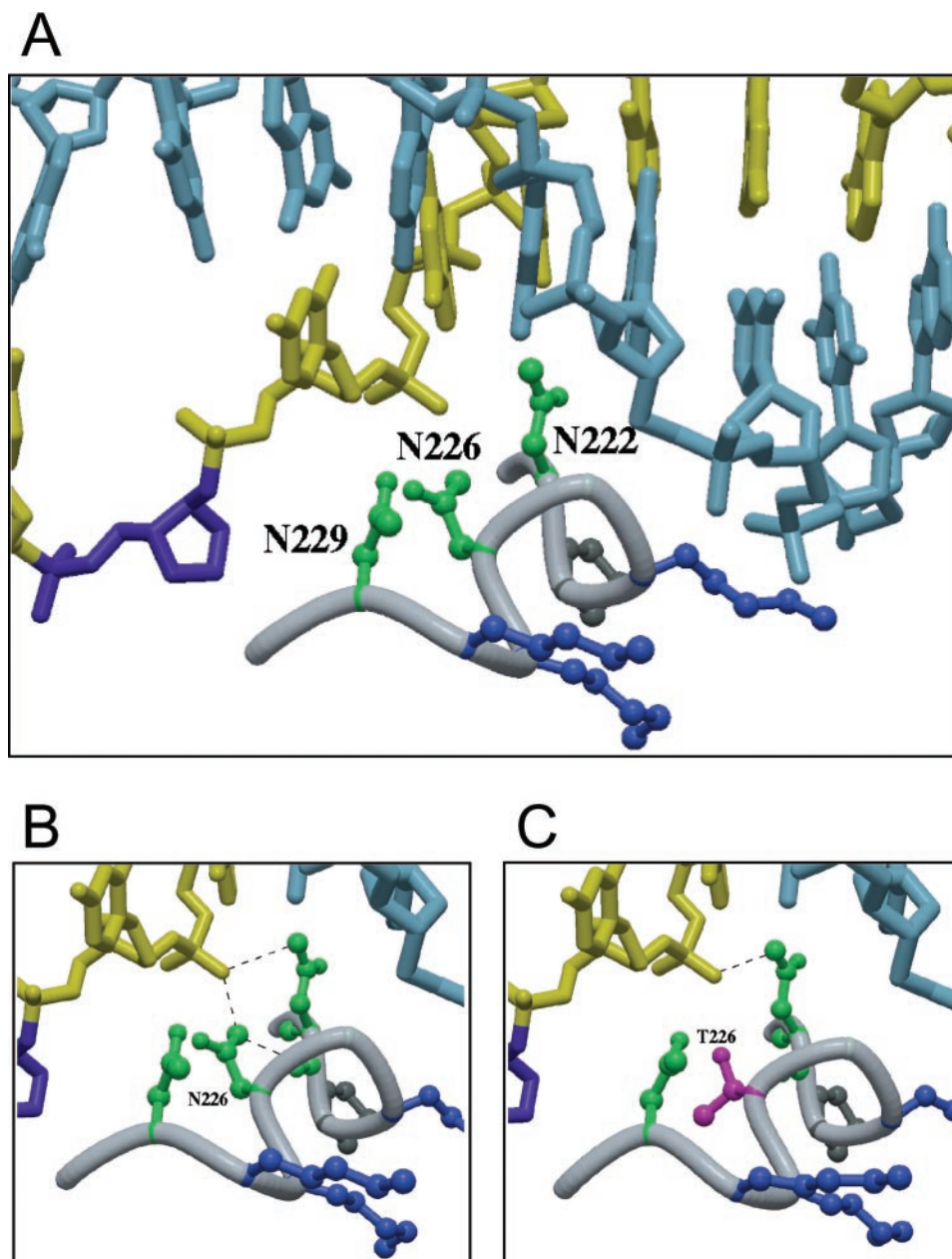


FIG. 1. The $\alpha 8$ loop in human APE1 bound to abasic DNA. *A*, the model shows the side chains of amino acids Asn-222 through Asn-229, based on the structure of a co-crystal of APE1 and an abasic-site containing, 15-bp oligonucleotide (2). In this complex, two asparagine residues, Asn-226 and Asn-222 contact phosphate residues in the abasic site-containing DNA strand 3' of the flipped out baseless residue. Asn side chains are colored *green*, Lys side chains are *dark blue*, Pro is *dark gray*, and Gly is *light gray*. The abasic site is colored *purple*, the remaining nucleotides in that strand, *yellow*; nucleotides in the opposite strand are *light blue*. The coordinate set (1DEW) was obtained from the Protein Data Bank (19). *B*, a close-up of wild-type Asn-226 shows hydrogen bonds from atom N $\delta 2$ of Asn-226 to the phosphate of guanine 39, the second base away from the abasic site, from N $\delta 2$ to the carbonyl oxygen of residue 222, and from N $\delta 2$ of Asn-222 to that phosphate oxygen. The two former distances are good hydrogen bond distances (2.6 and 2.8 Å, respectively, whereas the latter is weaker (3.3 Å). In the coordinate set 1DEW, the atom named O $\delta 1$ was oriented toward the phosphate; we renamed this atom N $\delta 2$ and the other O $\delta 1$ inasmuch as this seems more likely for hydrogen bonding. In another structure (1DE8) of APE1 complexed with a different, 11-bp abasic-site containing oligomer, N $\delta 2$ of Asn-226 forms a putative hydrogen bond with the analogous phosphate of the second base away from the abasic site, and N $\delta 2$ of Asn-229 is within hydrogen bonding distance of the phosphate immediately 3' of the abasic site (2, 19). *C*, a close-up of residue 226 mutated to threonine, in the same orientation, showing a lack of hydrogen bonds from that side chain. Possible hydrogen bonds from O $\gamma 1$ of the side chain are not well oriented, and the distances are greater than 3.3 Å. The homology modeling was carried out with tools available in the suite of programs in the "Molecular Operating Environment" available from the Chemical Computing Group, Montreal (see www.chemcomp.com). The residue was mutated to threonine, and all atoms of residues within 12 Å of the threonine residue, except the nucleotides, were energy minimized.

proteins were visualized on a Coomassie Blue R-250 (Bio-Rad Laboratories, Hercules, CA)-stained gel after 12.5% SDS-PAGE. The concentration of the purified proteins was determined by using a Bio-Rad Protein Assay kit based on the method of Bradford (Bio-Rad).

AP Endonuclease Assay—Synthetic oligonucleotide containing a tetrahydrofuran (F) that mimics an abasic site (5'-CTGGGTTTCTCCAA-TAACFGAATCCAACCTGGGCCGTAAC-3') and its complementary oligonucleotide (5'-GTTACCGGCCAGTTGGATTCCGTTATTGGAG-

AAACCCAG-3') were purchased from Invitrogen. The abasic oligonucleotide was 5'-end-labeled with [γ - 32 P]ATP and then hybridized with the complementary strand to form a double-stranded oligonucleotide containing an AP site. This AP-DNA would serve as a substrate for cleavage by APE1 proteins in the reaction buffer (50 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 100 mM NaCl, 1 mM DTT, and 0.1 mg/ml bovine serum albumin) at 37 °C for 10 min unless otherwise stated. The reaction product was resolved by electrophoresis in a 14% polyacrylamide gel

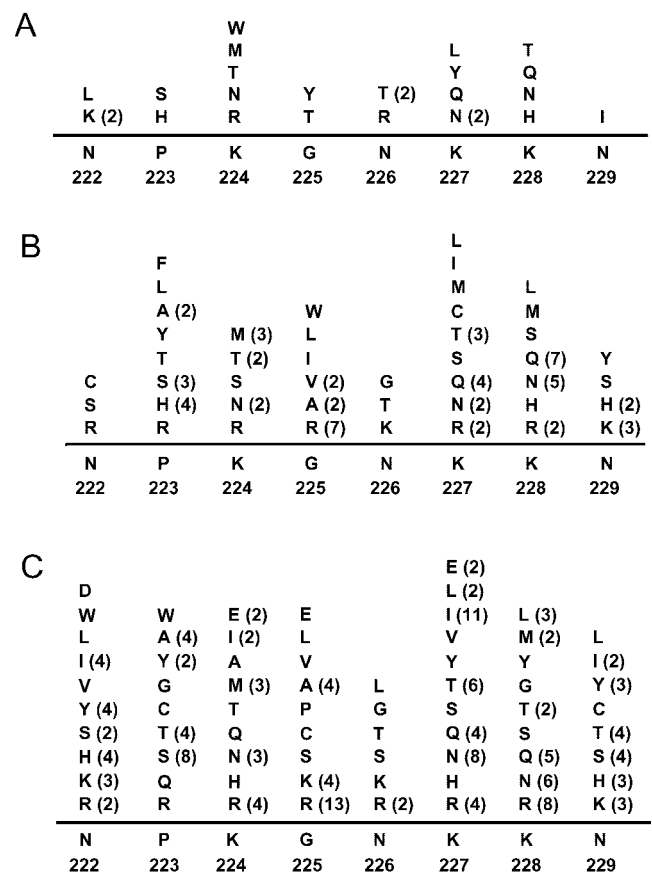


FIG. 3. Amino acid replacements in active mutants with single (A), double (B), and triple (C) mutations. Substituted amino acids are indicated above the wild-type sequence with basic, uncharged polar, nonpolar, and acidic residues listed in order from *bottom* to *top*. The number of times particular replacements were observed is indicated in *parentheses*. Mutants analyzed are 25 single-, 42 double-, and 63 triple-substitution clones.

acts with the major groove on the 3' side of the abasic site. A model illustrating interactions of the loop with abasic-site containing DNA is shown in Fig. 1A. The $\alpha 8$ loop mutant library was inserted into the bacterial expression vector pTrc-His2 that provides a histidine tag at the C terminus to facilitate purification of expressed proteins. Active mutants were obtained by transforming the library into AP endonuclease-deficient *E. coli* BW542 (*xth*⁻ *nfo*⁻) that lack ExoIII and endonuclease IV, and selecting for resistance to MMS. BW542 is highly sensitive to MMS (12), and we found that survival after MMS treatment can be up to five orders of magnitude lower than survival of the parental strain BW35 (see Fig. 4A). Transformation with wild-type APE1 enhanced survival of BW542 up to 1000-fold at a high MMS concentration (5 μ l/ml, 59 mM), although survival was \sim 100-fold lower than that exhibited by the parental strain (Fig. 4A). We exploited the ability of APE1 to complement the MMS sensitivity of BW542 to select for active mutants. Starting with a random library of two million clones, we carried out eight rounds of selection in the presence of increasing amounts of MMS. A gradual increase of MMS concentration in multiple rounds of selection allowed progressive enrichment for active mutants.

Analysis of Active Loop $\alpha 8$ Mutants—The final library of active mutants consisted of $\sim 2 \times 10^5$ members, each of which rendered *E. coli* resistant to 2 μM MMS (23.6 mM) in a 15-min treatment in the eighth round of selection. We randomly picked 187 clones from these active mutants for DNA sequence analysis. For comparison, we carried out DNA sequencing on 60

Creation and Genetic Selection of a Human APE1 Mutant Library—We created a human APE1 mutant library harboring random nucleotide substitutions at eight contiguous codons corresponding to the $\alpha 8$ loop, a DNA-binding region that inter-

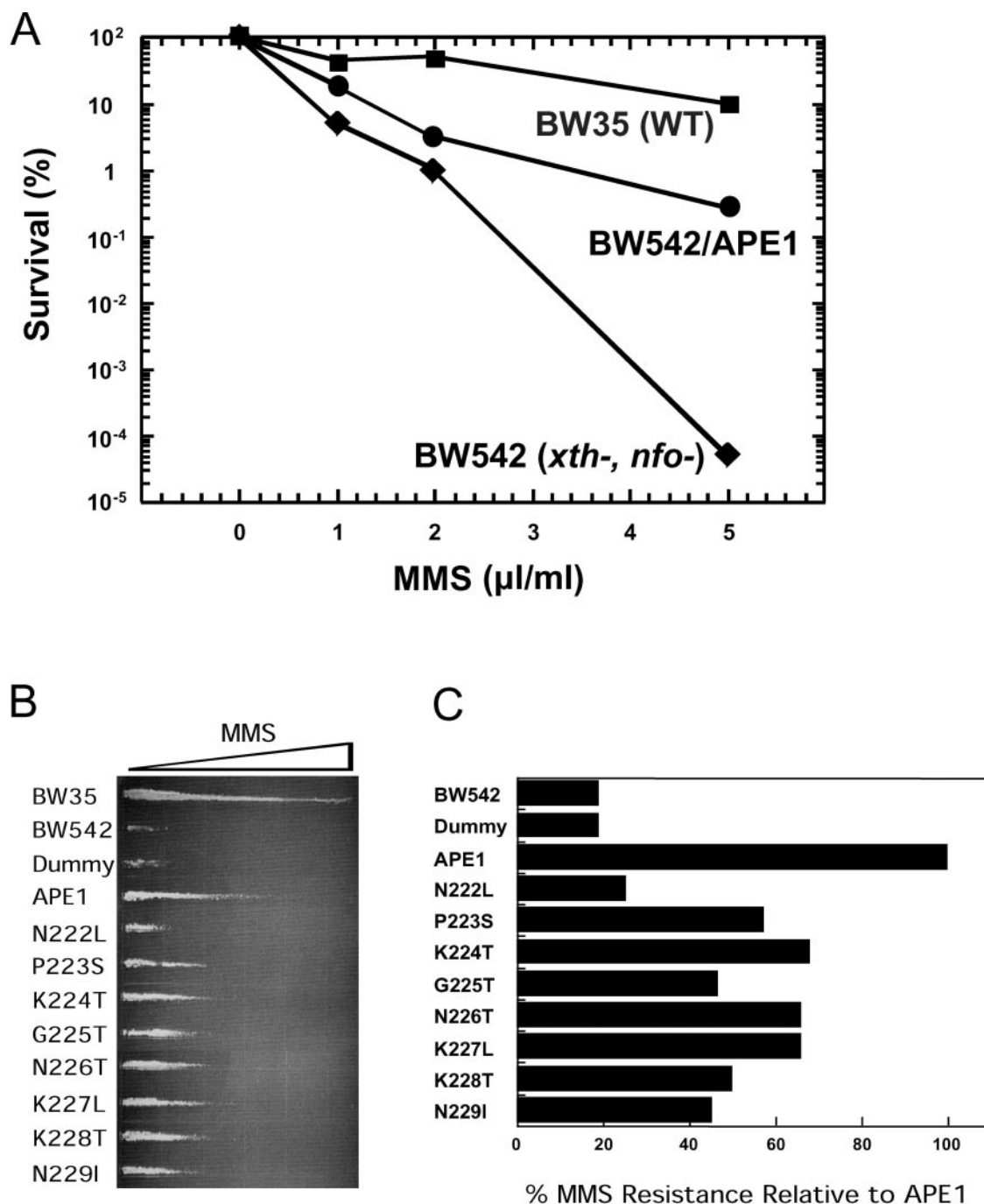


FIG. 4. **Complementation of MMS sensitivity in *E. coli* by APE1 loop $\alpha 8$ single mutants.** A, AP endonuclease-deficient *E. coli* strain BW542 (*xth*⁻ *nfo*⁻), the parental strain BW35, and BW542 transformed with plasmids encoding wild-type APE1 were grown to early log phase ($A_{600} = 0.3$) in LB broth. The bacterial cultures (1 ml) in LB broth containing the indicated concentrations of MMS were incubated at room temperature (23 °C) for 1 h. Following incubation, cells were diluted in LB and spread on LB plates; colonies were counted after overnight incubation at 37 °C. B, AP endonuclease-deficient *E. coli* strain BW542 (*xth*⁻ *nfo*⁻), the parental strain BW35, and BW542 transformed with plasmids encoding wild-type APE1, a dummy construct, and single mutants were grown on an MMS concentration gradient plate (0–1 μ l/ml MMS). Results are representative of three independent experiments. C, MMS resistance is measured as length of the streak and is indicated relative to that of wild-type APE1 (100%).

clones randomly picked from the original, unselected library. As shown in Fig. 2A, the average number of amino acid changes per APE1 DNA was reduced from 4.9 in mutants from the unselected library to 2.9 in the active mutants. This result indicates that only a limited number of amino acid substitutions was tolerated under the selection conditions. Among the 187 active clones, mutants with triple amino acid substitutions were most prevalent (34%), followed by double substitutions (23%), quadruple substitutions (20%), and single substitutions (13%).

Among the 60 unselected mutants, substitutions in the eight targeted amino acids included acidic, basic, uncharged polar, and hydrophobic residues.² No wild-type sequences were observed and ten mutants encoded a stop codon (Fig. 2B). Substitutions were distributed relatively evenly among the eight

² This analysis is based on RasMol's classification of amino acids in the molecular visualization tool RasTop 2.0 (www.inrp.fr/Acces/biotic/rastop/help/default.htm). Conservative substitutions are those involving amino acids in the same class.

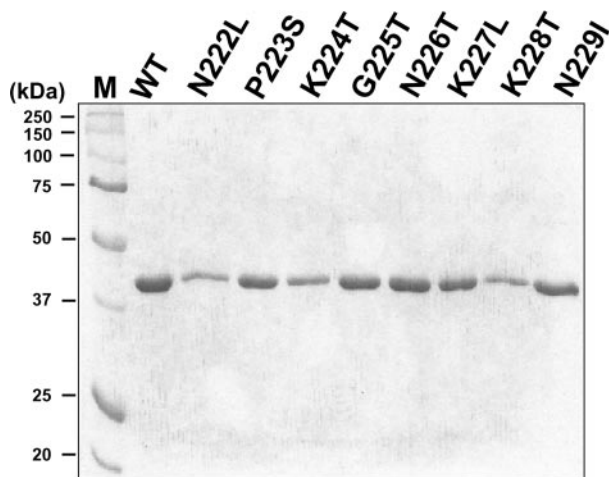


FIG. 5. SDS-PAGE analysis of purified APE1 proteins. Wild-type and singly substituted APE1 proteins were purified from *E. coli* extracts by nickel-affinity chromatography. Purified proteins and molecular weight standards were subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel, and the gel was stained with Coomassie Blue R-250.

randomized positions. Among the active clones, in contrast, no mutants containing a stop codon were observed, attesting to the efficacy of selection. Acidic substitutions were not observed in the single and double mutants, and only six of the triple mutants harbored a lone acidic residue, indicating that the basic character of the loop is important for function.

The spectra of single, double, and triple substitutions in the 187 active mutants are presented in Fig. 3, A, B, and C, respectively. Only one wild-type clone was observed, presumably representing a false-positive, in which Asn-226 was encoded by AAT rather than the wild-type triplet AAC. The spectrum of replacements in the 25 single mutants indicates that the $\alpha 8$ loop tolerates amino acid changes at every residue. The replacements in the single and double mutants, within each group and in combination, are about one-quarter basic and one-half uncharged polar amino acids.² This pattern of substitution reflects the fundamentally basic and polar nature of the wild-type loop. Considering only the single and double mutants, Asn-226, Asn-222, and Asn-229, exhibited the lowest proportion of total substitutions (5.5%, 5.5%, and 7%, respectively). The remaining wild-type residues displayed higher proportions, varying from 13% to 20%. Asn-222, Asn-226, and Asn-229 also tolerated the narrowest spectrum of replacements (five, four, and five different substitutions, respectively), the other residues exhibiting from six to ten different replacements. These data indicate that Asn-222, Asn-226, and Asn-229 are the least mutable residues in the loop; each is postulated to participate in hydrogen bonding with the DNA backbone downstream of the flipped out AP site, stabilizing the APE1-DNA complex for catalysis (2). Of the three asparagines, the proportion of conservative substitutions was highest for Asn-226. Moreover, among all 187 active mutants, Asn-226 was replaced in fewer of the mutants (16%) than any other wild-type residue. We infer that Asn-226 may have particular functional significance, perhaps related to its postulated role in H-bonding. Interestingly, the amino acid sequence of loop $\alpha 8$ is identical in mammals, including human, bovine, and mouse, and is highly conserved with respect to the *Drosophila* homolog Rrp1, the only differences being substitution of Asn and Thr at positions corresponding to Gly-225 and Lys-227 in APE1, respectively. In contrast to this evolutionary conservation, multiple mutations in the $\alpha 8$ loop are permitted under our selection scheme. It is important to note in this regard that mutants

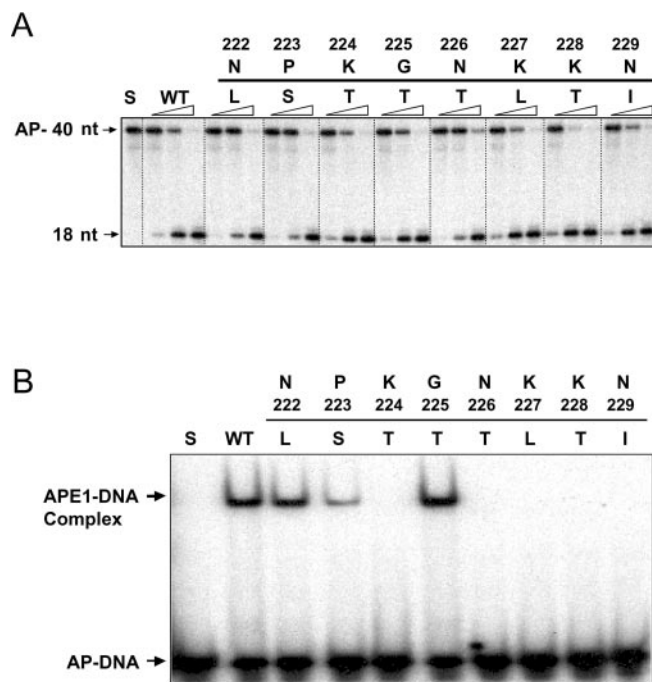


FIG. 6. Effect of loop $\alpha 8$ single mutations on AP endonuclease and AP-DNA binding activities of APE1. A, the AP endonuclease activity of purified wild-type (WT) and mutant APE1 was assayed in 10- μ l reaction mixtures containing 10 nM ³²P-AP-DNA (40 bp) and 0.001, 0.01, or 0.1 nM APE1. The mixtures were incubated at 37 °C for 10 min, and reaction products were resolved in a 14% denaturing gel. The 18-nt fragment resulting from incision and the uncut 40-nt AP oligonucleotide were visualized by autoradiography. S, substrate alone. B, the AP-DNA-binding activity of APE1 proteins was determined by electrophoresis mobility shift assays. APE1 protein (100 nM) was incubated with AP-DNA (10 nM) in a 10- μ l reaction in the absence of MgCl₂ at room temperature for 20 min, followed by a 10-min incubation at 4 °C. The APE1-DNA complex was separated from AP-DNA by electrophoresis in a 6% polyacrylamide gel and visualized by autoradiography. Results are representative of three separate experiments.

possessing as little as 10% of the wild-type incision activity were selectable (data not shown), even though all active mutants survived multiple rounds of exposure to MMS. This observation suggests that AP endonuclease activity is present in cells in excess, in accord with increased demand during periods of oxidative stress.

Effects of Single Amino Acid Substitutions on APE1 Activity in Vivo—Analysis of our active mutant library after eight rounds of MMS selection indicates that the $\alpha 8$ loop is mutable and tolerates multiple substitutions. To gain insight into the role of the loop in AP site binding and AP endonuclease activity, we analyzed the effect of amino acid substitutions within the loop structure. It is difficult to infer changes in the structure of mutants that contain multiple substitutions. Therefore, we chose a group of eight single mutants for analysis that includes a replacement at each of the targeted residues; most of the replacements result in loss of putative hydrogen bonds (2, 19) or a reduction in net charge. We measured the capability of the mutants to complement the MMS sensitivity of AP endonuclease-deficient *E. coli* BW542. Bacteria harboring mutant APE1 were grown to stationary phase and applied in a straight line on the surface of plates containing a linear MMS gradient. After overnight incubation at 37 °C, complementation was visualized by the extent of growth along the line of increasing MMS concentration, as illustrated in Fig. 4A. In comparison with the parental strain BW35, the repair-deficient strain BW542 (*xth*⁻ *nfo*⁻) was highly sensitive to MMS. This sensitivity was not reduced by transformation with a dummy vector (*i.e.* a vector with an inactive insert that encodes IPIRAKNN

TABLE I
Kinetic analysis of the APendonuclease activity of APE1 mutants

	WT	APE1 mutants							
		N222L	P223S	K224T	G225T	N226T	K227L	K228T	N229I
k_{cat} (s ⁻¹)	1.1	0.39	0.52	1.1	1.2	1.1	2.1	4.8	2.0
K_m (nM)	9.4	10	27	13	11	55	25	40	31
k_{cat}/K_m (s ⁻¹ nM ⁻¹)	0.12	0.038	0.019	0.084	0.11	0.019	0.083	0.12	0.065

rather than the wild-type NPKGNKKN). In contrast, wild-type APE1 partially complemented the host repair deficiency. All mutants tested complemented BW542, which is not surprising, because they were selected in eight rounds of MMS treatment. However, none complemented as well as wild-type APE1 (Fig. 4, *B* and *C*). The extent of MMS resistance was similar among the mutants, with the exception of N222L, which exhibited much weaker complementation. These data show that even though loop $\alpha 8$ is highly mutable, a single amino acid substitution can compromise APE1 function *in vivo*.

Effect of Amino Acid Replacements on Incision and Binding of AP-DNA—To analyze the effects of amino acid substitutions in the $\alpha 8$ loop on cleavage and binding of AP site-containing DNA, we expressed and purified the eight mutant proteins described above. The mutant proteins as well as wild-type APE1 were isolated from *E. coli* extracts by Ni^{2+} affinity chromatography, and the purity of the final preparations was examined by SDS-PAGE. Each preparation showed a single band and was estimated to be at least 95% homogeneous; the APE1 proteins, fused with a *myc* epitope and a hexahistidine tag at the C terminus, migrated with mobility corresponding to ~ 45 kDa (Fig. 5). We measured AP endonuclease activity in a gel-based assay that employs a ^{32}P -labeled, synthetic oligonucleotide containing a tetrahydrofuran residue at the center of the duplex to mimic an AP site. After cleavage by AP endonuclease activity to generate a nick 5' of the AP site, the reaction mixtures containing the DNA duplexes (40 bp) were run on a denaturing polyacrylamide gel, and the cleavage product (18-nt single-stranded DNA) was visualized and quantified based on the 5'- ^{32}P -end label. As shown in Fig. 6A, wild-type APE1 and all eight mutants incise AP-DNA in a concentration-dependent manner. Steady-state kinetic parameters, determined in the gel-based assay, are summarized in Table I. All mutants other than G225T exhibited changes in the Michaelis-Menten constant, K_m , and/or the catalytic rate constant, k_{cat} . Mutation in the four C-terminal amino acids was associated, overall, with greater increases in K_m than mutation in the N-terminal residues, perhaps reflecting closer proximity to the AP site and greater importance in binding of AP-DNA.

The binding of wild-type and mutant APE1 to AP-DNA was assessed in electrophoretic mobility shift assays, and bound APE1-DNA complexes were visualized on gels by autoradiography. Overall, the C-terminal mutations exhibited greatly reduced binding relative to the N-terminal mutations (Fig. 6B). However, there was no fixed relationship between K_m , measured in activity assays, and binding in the gel shift assay. For example, K224T exhibited only a minor increase in K_m but did not display detectable binding. In contrast, P223S, with a higher K_m than K224T, visibly bound AP-DNA, albeit to a lesser extent than wild-type. These results could be due to divalent cations that are present in the enzymatic assay but not in the binding assay.

DISCUSSION

We have created a human APE1 mutant library harboring amino acid substitutions in the eight-residue $\alpha 8$ loop that interacts with the DNA major groove on the 3'-side of the AP site. Fig. 1A shows a model of the loop illustrating interaction with

the abasic site-containing DNA strand. Although the amino acid composition of the loop is highly conserved from flies to mammals (18), our results indicate that every residue is replaceable and that active mutants can be selected that confer MMS resistance to AP endonuclease-deficient *E. coli*. Of the eight residues, the three asparagines (Asn-222, Asn-226, and Asn-229) that are postulated to form hydrogen bonds with the DNA backbone (2, 19), exhibited least mutability, Asn-226 being lowest. The amino acid replacements in active mutants have predominantly basic or uncharged polar side chains, in accord with the characteristics of the wild-type loop and the interaction of the loop with AP-DNA observed in crystal structures. Multiple substitutions within this motif are tolerated, although all mutants retained a net positive charge (Fig. 3 and data not shown). In addition to altering electrostatic charge or hydrogen-bonding potential, the substitutions we observed may cause conformational changes that affect the function of the loop. Despite plasticity of the $\alpha 8$ loop with respect to amino acid replacements, we found that substitution at every position can compromise complementation of MMS sensitivity in *E. coli* (Fig. 4, *B* and *C*). These results suggest that the $\alpha 8$ loop may be important for binding and/or incision of AP site containing DNA.

We purified APE1 from eight active mutants that contain a single substitution at each of the $\alpha 8$ loop positions and characterized their AP site incision activity and binding to an AP site containing oligomer. Steady-state kinetic analysis showed that a single replacement can change the parameters of AP site cleavage. In general, the C-terminal half of the loop (Asn-226, Lys-227, Lys-228, and Asn-229), which is positioned closer to the AP site and harbors two of the three asparagines that contact the DNA backbone (2), showed the largest effects on K_m . For example, substitution of Asn-226 with Thr resulted in a 6-fold increase of K_m relative to wild-type APE1 (Table I). A model of Asn-226 in APE1 bound to a 15-bp oligonucleotide (Fig. 1B) shows predicted hydrogen bonding of Asn-226 and Asn-222 to the DNA downstream of the abasic site. In a second complex of APE1 with a different, 11-bp oligonucleotide, Asn-226 and Asn-229 form predicted hydrogen bonds with the DNA backbone (2, 19). Substitution of threonine in the N226T mutant (Fig. 1C) results in loss of predicted hydrogen bonding that may account, at least in part, for our observations. Substitution at Lys-227, Lys-228, and Asn-229 also increased K_m 3–4-fold. As shown in Fig. 6B, these elevated K_m values were reflected in reduction of AP-DNA binding affinity determined by electrophoretic mobility shift assay. In general, substitutions in the C-terminal residues closer to the AP site (Asn-226, Lys-227, Lys-228, and Asn-229) had greater effect on binding (Fig. 6B). It should be noted that changes in K_m do not necessarily parallel changes in binding. Thus, K224T showed a less than 2-fold increase in K_m but exhibited no detectable binding. In contrast, P223S had a 3-fold increase in K_m but still bound to AP-DNA, although binding was weaker than that observed for wild-type APE1. The varying relationship may reflect the fact that the binding assay was carried out in the absence of Mg^{2+} ions, and this could be important in APE1/AP-DNA association and dissociation (14).

Site-directed mutagenesis based on phylogenetic considerations and on crystallographic analysis has identified several essential amino acids at the active site of APE1 (13–16). These studies have focused largely on conserved amino acids or catalytic residues. Our random mutagenesis study targeting a dynamic DNA-interacting loop provides insight into how alterations in a regional peptide sequence can affect substrate binding as well as catalytic activity. Our work describing the *in vivo* and *in vitro* effects of mutation in the $\alpha 8$ loop supports and extends the conclusion of x-ray analysis that the loop is important for binding of AP-DNA and AP site incision.

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