Effects of Backbone Contacts 3′ to the Abasic Site on the Cleavage and the Product Binding by Human Apurinic/Apyrimidinic Endonuclease (APE1)†

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ABSTRACT: The mammalian apurinic/apyrimidinic (AP) endonuclease (APE1) is a multifunctional protein that plays essential roles in DNA repair and gene regulation. We decomposed the APEs into 12 blocks of highly conserved sequence and structure (molegos). This analysis suggested that residues in molegos common to all APEs, but not to the less specific nuclease, DNase I, would dictate enhanced binding to damaged DNA. To test this hypothesis, alanine was substituted for N226 and N229, which form hydrogen bonds to the DNA backbone 3′ of the AP sites in crystal structures of the APE1/DNA complex. While the cleavage rate at AP sites of both N226A and N229A mutants increased, their ability to bind to damaged DNA decreased. The ability of a double mutant (N226A/N229A) to bind damaged DNA was further decreased, while the V_{max} was almost identical to that of the wild-type APE1. A double mutant at N226 and R177, a residue that binds to the same phosphate as N229, had a significantly decreased activity and substrate binding. As the affinity for product DNA was decreased in all the mutants, the enhanced reaction rate of the single mutants could be due to alleviation of product inhibition of the enzyme. We conclude that hydrogen bonds to phosphate groups 3′ to the cleavage site is essential for APE1's binding to the product DNA, which may be necessary for efficient functioning of the base excision repair pathway. The results indicate that the molego analysis can aid in the redesign of proteins with altered binding affinity and activity.

DNA in all living organisms is continuously attacked by reactive oxygen species (ROS)[1] and environmental genotoxicants such as DNA alkylating reagents (*1*−*3*). These reagents generate abnormal bases, apurinic/apyrimidinic (AP) sites, and DNA strand breaks, which are either toxic or mutagenic. To maintain their genomic integrity, all organisms have a similar base excision repair (BER) pathway to remove damaged sites in DNA (*4*, *5*). In the first step of this streamlined pathway (*6*, *7*), DNA glycosylases cleave the N-glycosylic bonds of abnormal bases to generate either AP sites or DNA strand breaks by β- or $\beta\delta$-elimination reaction (*8*, *9*). Next, AP endonucleases (APEs) generate 3′-OH termini that serve as primers for subsequent gap filling reactions by DNA polymerases (*7*), followed by strand-sealing reactions by DNA ligases (*10*).

APE1, a key player in the mammalian BER pathway, is an essential protein; embryos of APE1 homozygous knockout mice died during early embryonic development (E3.5-E5.5) (*11*−*13*). In addition to its role in DNA repair, APE1 also functions as a redox gene regulator to activate such critical transcriptional factors as cJun/cFos, p53, and HIF1-α (*14*−*16*). APE1 is also a transcriptional repressor of the parathyroid hormone gene and possibly of the APE1 gene itself (*17*−*19*). The redox mechanism, which apparently requires a Cys residue in mammalian APE1, has not been completely established (*20*). The mechanism of the repressor function has been analyzed and reported to involve acetylation of N-terminal Lys residues (*21*).

The residues of APE1 that are essential for cleavage of DNA at AP sites have been identified by biochemical and structural studies (*22*−*28*), in particular by crystal structures of APE1 bound to substrate DNA (*28*). Most of the residues known to be required for hydrolysis and Mg^{2+} coordination, such as E96, D210, and H309 were easily identified by their conservation in the sequences of APE family members, including *Escherichia coli* exonuclease III (Xth). In addition to conserved residues, a structural study revealed that Arg 177 (R177) penetrates the substrate DNA through the major groove, and forms a hydrogen bond to the DNA backbone (*28*). The residue is not required for the AP endonuclease activity, but crucial for product DNA binding (*28*), which is believed to be a common feature among many BER enzymes for a coordinated repair process. It is possible that other residues are also required for APE1's high affinity for the cleaved DNA. However, simple sequence conservation analysis does not reveal how APE1 specifically recognizes the substrate and cleaved DNA.

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[1] Abbreviations: AP site, apurinic/apyrimidinic site; APE, AP endonuclease; BER, base excision repair; CD, circular dichroism; *E. coli*, *Escherichia coli*; EMSA, electrophoresis gel retardation assay; ROS, reactive oxygen species; Xth, exonuclease III.

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We used a different approach to the problem, which involved identifying discrete conserved areas in a sequence alignment of many APEs from diverse sources with our web-based MASIA tool (*29*). Ten of the 12 conserved motifs so identified were then shown to be molecular building blocks, or "molegos", as they formed distinct secondary structural elements in the crystal structures of Xth and APE1 alone and in complex with the substrate DNA (*29*). Five molegos are common to DNase I (*29*). Comparison of the crystal structures of complexes of human APE1 and DNase I with their DNA substrates indicated that these molegos were essential components for the nucleolytic activity of both proteins. Indeed, some of the molegos are shared by distantly related enzymes such as inositol-5′-polyphosphate phosphatases (*29*), that catalyze phosphorolytic cleavage, but have different substrate specificity than APE1. This modular approach, in which blocks of functional units (molegos) are first identified and then evaluated in detail, is an efficient method to determine the molecular basis of functional diversity among similar enzymes. By comparing APE1's side chain composition to that of the less specific endonuclease, DNase I, we were able to identify a region of APE1 that contributes to product binding specifically. The present study reports that mutations in these regions in APE1 affect both substrate and product binding.

EXPERIMENTAL PROCEDURES

Construction of Mutants by PCR. The source of the wild-type (WT) and R177A human APE1 cDNA was described earlier (*30*). Other missense mutants, including N226A, N229A, N226A/N229A, and R177A/N226A, were created by PCR-cloning with specific primers. Briefly, a vector primer and an oligonucleotide primer (5′ CCC CTT AGG GTT GCG AAG GTC AAT 3′), which anneals just upstream of the N226 or N229 site of the hAPE1 cDNA to introduce a *Bsu*36I site, was used for PCR-cloning of the upstream part of the hAPE1 cDNA using a pTrcHis2-TOPO cloning kit (Invitrogen). Then primers for N226A (5′ CGC AAC CCT AAG GGG GCG AAA AAG AAT GCT GGC TTC 3′), N229A (5′ CGC AAC CCT AAG GGG AAC AAA AAG GCG GCT GGC TTC 3′), and N226A/N229A (5′ CGC AAC CCT AAG GGG GCG AAA AAG GCG GCT GGC TTC 3′) were used to amplify the downstream part of the hAPE1 cDNA with a vector primer, and cloned into the pTrcHis2 derivative using *Bsu*36I. The DNA containing missense mutations were finally cloned into the pET15b protein expression vector (Novagen) as described previously (*22*). The DNA sequences of the mutant cDNA were confirmed by UTMB's Protein Chemistry Core laboratory.

Purification of Proteins. APE1 proteins were expressed and purified by the method described earlier (*22*) with slight modifications. Briefly, *E. coli* BL21/codon plus was transformed with the pET15b-derivatives, and the bacteria cultures (500 mL in LB broth) were grown to OD600 of 0.3−0.5. Then the proteins were induced with 0.5 mM IPTG at 16 °C for 14−16 h (*31*, *32*). Cells were suspended in a suspension buffer containing 20 mM Tris (pH 8.0) and 1 M NaCl, and broken by sonication. After centrifugation, the extracts were applied to a 3-mL column of Ni−NTA. The column was washed with 18 mL of the suspension buffer and with the same buffer cotaining 20 mM imidazole. The APE1 protein was then eluted with 10 mL of the suspension buffer with 200 mM imidazole, and then dialyzed against 20 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 1 mM dithiotreitol (DTT). The histidine tag peptides were removed by thrombin at 4 °C for overnight incubation, and the fractions were further purified to homogeneity with SP−Sepharose column chromatography using the AKTA purifier system (Pharmacia). The final fractions were dialyzed against 20 mM Tris (pH 8.0), 300 mM NaCl, 1 mM DTT, and 50% glycerol, and stored at −20 °C.

Circular Dichroism (CD) Spectrum. The secondary structure of the APE1 proteins were monitored with an AVIV 62 DS circular dichroism spectropolarimeter. The far-UV CD spectra (195−260 nm) were obtained in fused quartz cuvettes with 0.1-cm path length with protein solutions in 20 mM Tris-Cl (8.0) and 150 mM NaCl. Each spectrum was recorded with a 1-nm increment. For each sample (with a concentration of about 200 μg/mL), three repetitive scans were obtained and averaged. At least two independent experiments were carried out.

AP-Endonuclease Activity. For kinetics study, a 43-mer oligonucleotide containing the AP site analogue, tetrahydrofuran, was used as described previously (*22*, *33*). The oligo was 5′-end-labeled with [γ-^{32}P]ATP and purified by gelfiltration using Sephadex G25. The substrate for the AP-endonuclease assay was prepared by annealing the 43-mer oligonucleotide (Midland Corp) with the reverse complementary oligonucleotide (*22*), followed by purification using nondenaturing polyacrylamide gel electrohoresis (PAGE). APE1 proteins (22 pM) were mixed with the substrates with concentration (indicated in Figure 3 as [S]), and incubated at 37 °C for 0−4 min. The reaction within this time period exhibited a linear increase of the product DNA (data not shown). The reactions were stopped by the addition of the stop buffer (10 mM EDTA in 88% formamide solution with 0.05% bromophenol blue) and kept on ice until the samples were analyzed in 8% PAGE containing 8 M urea to separate the substrate DNA from the cleaved product. The gels were dried and then analyzed with a phosphorescence detection system (Storm, Molecular Dynamics). The kinetics data were fitted by nonlinear least-squares regression to obtain V_{max} and K_m using the Michaelis−Menten equation.

Electorophoretic Mobility Shift Assay (EMSA). The 5′-[^{32}P]-labeled 43-mer substrate DNA (about 25 pM) was incubated with purified WT-APE1 and the indicated mutants thereof (0−500 nM as shown in Figure 4) in the binding buffer containing 25 mM HEPES, 50 mM NaCl, 4% Ficoll, 1 mM DTT, 2.5 mM EDTA for 30 min at 25 °C. The samples were then run in nondenaturing 5% PAGE in the buffer containing 6.7 mM Tris, 3.3 mM sodium acetate, 1 mM EDTA at 4 °C. The gels were dried and then analyzed with the Storm system. The data were fittedusing Sigmaplot in the equation:

$$f = \frac{[E]}{[E] + K_d}$$

where f equals to the ratio of bound DNA to total DNA ($[DNA]_B/[DNA]_T$), $[E]$ is the concentration of the protein, and K_d is the dissociation constant. The protein concentration was in excess of that of the bound DNA. APE1's binding to the product DNA (after the hydrolysis reaction) was observed with the AP site-containing DNA (either the 43-mer oligo-

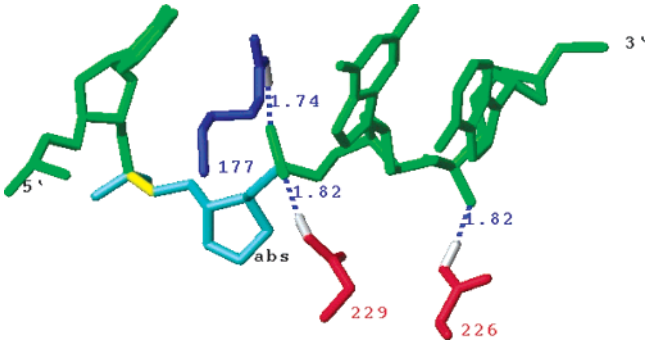

FIGURE 1: The hydrogen bonding network 3′ to the AP site of damaged DNA formed by residues N226, N229, and R177 of APE1. The contacts (and their length in angstroms) shown with the structure of the damaged DNA are taken from the 1DE8.pdb coordinate file (*28*). Colors: N226/N229 red, R177 blue, abasic site turquoise, DNA green, hydrogen involved in H-bonds gray, and the bond to be cleaved yellow.

nucleotide or 5′-Cy5 labeled 26-mer, IDT technology) in the same buffer containing 2 mM $MgCl_2$ and no EDTA. The sequence of the 26-mer DNA is 5′ TCCTCAGTTTCACTX-CTGCACCGCAT, where X denotes tetrahydrofuran.

RESULTS

N226 and N229 Form Hydrogen Bonds to DNA Backbone Downstream to AP Sites. Of the molegos specific to APE1, two molegos were in contact with the two bases 3′ to the AP site on the damaged strand in the cocrystal structure of the protein with its DNA (*29*). One residue in molego 7, N226, formed hydrogen bonds to the phosphate backbone in three cocrystal structures, while N229, from a neighboring, less conserved sequence area, was within hydrogen bonding distance in only one (1DE8, Figure 1). Although an exact sequence alignment between APE1 and Xth in this area is not possible, N226 probably corresponds to N167 in the latter. In the 1DE8 crystal structure of hAPE1 bound to the substrate DNA, N229 binds to the same phosphate, one base downstream from the AP site, as R177, mutation of which in previous studies (*28*) was shown to increase V_{max} but decrease binding to its substrate.

N226 and N229 Mutants Showed Higher V_{max} and K_m Compared to the WT Protein. To examine the importance of these contacts in APE1 catalysis, we constructed alanine substitution mutants, namely, N226A, N229A, and the double mutant N226A/N229A. These mutants and the WT protein were soluble when expressed in *E. coli* and could be purified to homogeneity by the same protocol (Figure 2). Both Asn residues are on the surface of the protein, so we expected no major conformational change. This was confirmed by circular dichroism (CD) spectra (Figure 2B). The spectra for the mutant proteins were essentially identical to that of the WT, confirming that their effect was only local and did not affect the global fold of the protein. The spectra contained a broad minimum centered at 210 nm, consistent with the primarily β-sheet structure of APE1. There was also a shoulder at 225−230 nm spectra for all APE1 proteins, which could reflect optical activity from exposed aromatic side chains (*34*).

Enhanced APE Activity of the N226A and N229A Mutants. Because of their direct interaction with both substrate and product DNA in the cocrystal structures (*28*), we inferred

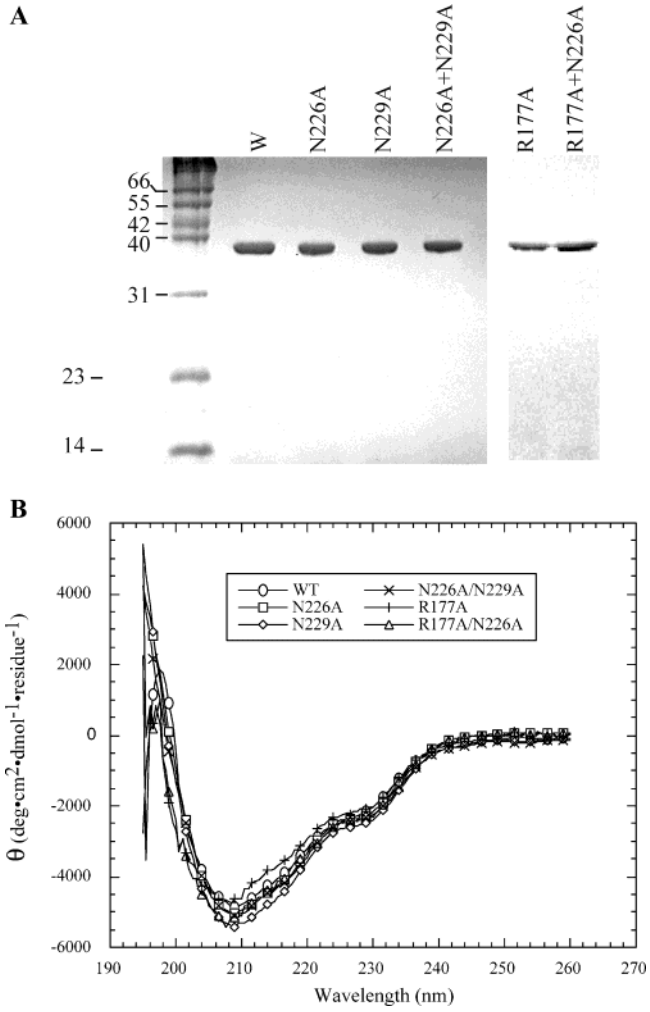

FIGURE 2: Purification of the WT and mutant APE1 proteins. (A) 12% SDS/PAGE of the purified proteins stained with Coommassie blue. Protein amount: 2 μg of the WT (W), N226A, N229A, and N226A/N229A; 1 μg of R177A, and R177A/N226A (B) CD spectra of APE1 proteins including the WT, R177A, N226A, N229A, N226A/N229A, R177A/N226A.

that the N226A and N229A mutations would affect APE1's affinity for the damage-containing DNA. We thus examined their AP endonuclease activity compared with the WT protein, to probe their influence on the DNA binding. To determine the kinetics parameters of the APE1 proteins for the AP endonuclease activity, we used tetrahydrofuran (THF) (*22*, *28*, *30*) because of its stability. The intact AP site is easily cleaved spontaneously via β-elimination reaction, and thus is not suitable for accurate quantitation of the cleavage reaction. A previous report also showed that APE1 is able to cleave THF with a similar efficiency to the intact AP sites (*35*). A 5′-end labeled oligonucleotide (43-mer) containing THF was incubated with the purified proteins, and the AP endonuclease reactions were assayed after denaturing gel electrophoresis which separated the cleaved DNA from the uncut substrate DNA (Figure 3, Table 1). The N226A, N229A, and N226A/N229A double mutant showed higher specific activity than the WT protein. The V_{max} of the single missense mutant proteins were more than 2-fold higher than the WT, and the double mutant also showed a slightly higher V_{max} (Table 1). However, all three mutants had significantly higher K_m than the WT protein. While the K_m was 4- and

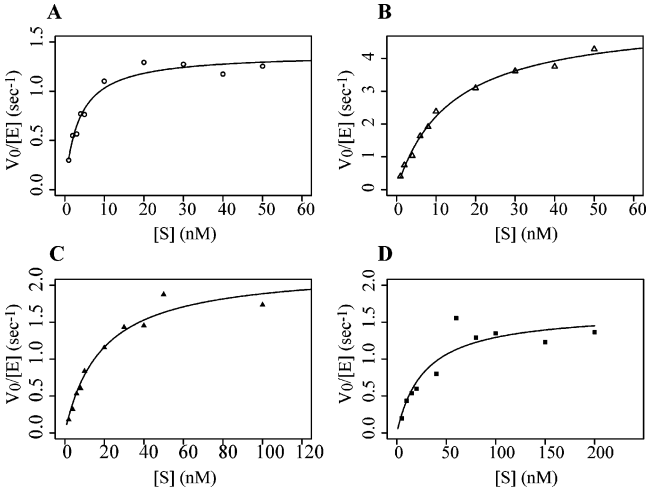

FIGURE 3: Effect of N226 and N229 on the APE activity. AP endonuclease activity for the WT APE1 (A), N226A (B), N229A (C), and N226A/N229A (D) mutants. The 5′-^{32}P-labeled 43-mer oligonucleotide was incubated with reaction conditions described in Experimental Procedures. [S]: substrate concentration (nM); v_0/[E]: cleaved products released by APE1 (s^{-1}).

Table 1: Kinetics of APE1 Proteins for AP-Endonuclease Activity[a]

	K_m (nM)	V_{max} (s^{-1})
WT	3.4 ± 0.48	1.38 ± 0.05
N226A	13.6 ± 1.21	5.26 ± 0.18
N229A	18.4 ± 3.4	2.28 ± 0.16
N226A/N229A	27.8 ± 10.0	1.65 ± 0.18

[a] 5′-^{32}P-labeled 43-mer oligonucleotide was used in the standard enzyme assay described in Experimental Procedures.

5.4-fold higher for the N226A and N229A mutants, respectively, the K_m of the N226A/N229A mutant was more than 8-fold higher, indicating that binding to the substrate was progressively weakened by removing residues that could form the hydrogen bond network. These data are similar to results obtained for the R177A mutant, which also showed a higher V_{max} /K_m values than that of the WT protein (*28*). Therefore, we tested whether the loss of the Arg residue would further affect the activity. The double mutant R177A/N226A, which was completely soluble and thus purified as the single missense mutant proteins, showed a drastic decrease in its specific activity (9×10^{-2} s^{-1}), 1/16 of the WT APE1.

Recognition of the Substrate and Product DNA by the APE1 Proteins. All the mutants were then tested for their ability to bind substrate DNA in the electrophoretic mobility shift assay (EMSA). The 43-mer THF-containing oligonucleotide was incubated with the WT and the mutant APE1 proteins. The binding reaction contained 2 mM EDTA and no divalent cation (Mg^{2+}), an essential cofactor for the APE activity, so that the APE1 binding occurred to the substrate DNA (*36*). All the mutants had lower affinity for the damaged DNA substrate than the wild type, consistent with their increased K_m values in the first experiments (Figure 4, Table 2). The affinity for the substrate DNA of the double mutant, R177A/N226A, was drastically decreased (Figure 4, Table 2). These data suggest that all three residues, R177, N226, and N229 contribute to proper substrate binding by APE1, and underscore the importance of the H-bonding network formed by these residues.

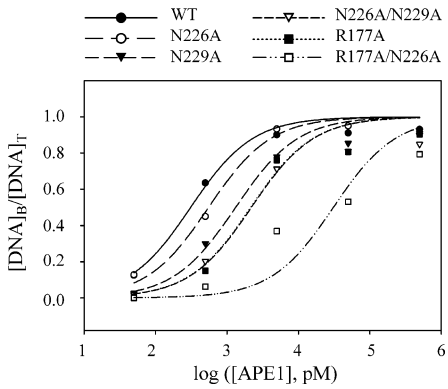

FIGURE 4: Effects of the missense mutations on substrate DNA binding. Electrophoresis mobility shift assay carried out with the THF containing 43-mer and APE1 proteins. Ratio of bound ($[DNA]_B$) to total DNA ($[DNA]_T$) was plotted as a function of enzyme concentration (indicated as log [APE1]).

Table 2: Affinity of APE1 Proteins for DNA with an AP Site[a]

protein	K_{d-app} (nM) ± STD
WT	0.31 ± 0.07
N226A	0.55 ± 0.12
N229A	1.33 ± 0.38
N226A/N229A	2.20 ± 0.87
R177A	2.15 ± 0.78
R177A/N226A	31 ± 16.5

[a] Values calculated based on Figure 4 using Sigmaplot.

Binding of Mutant and wt APE1 Protein to Cleaved Product DNA. The fact that APE1 formed a stable complex with a cleaved DNA and Mn^{2+} in the crystallographic study (*28*) suggests that APE1 may still interact with the DNA after its reaction and thus is not released efficiently from the DNA. Others have suggested that such binding may be necessary to ensure that the product is efficiently passed to the subsequent enzymes in the base excision repair pathway (*28*, *37*, *38*). Reduced product binding could explain the higher specific activity of the single missense mutants, as weakening APE1's affinity for the product DNA could enhance turnover. Thus, we carried out gel retardation assays for APE1/product DNA complexes to probe the importance of the residues for the product binding. An oligonucleotide containing an AP site was mixed with the purified APE1 proteins in the presence of 2 mM $MgCl_2$ and no EDTA, a condition in which APE1 is fully active. Specific binding of product of the mutants and WT APE1 was followed by gel electrophoresis (Figure 5). All the mutant APE1 proteins had decreased affinity for the cleaved DNA, and so released their products after cleavage (Figure 5). Denaturing gel electrophoresis which separates the substrate DNA and the cleaved DNA confirmed that the substrate DNA was completely cleaved by all APE1 proteins, confirming that the complex in this condition was formed between APE1 and the cleaved DNA (Figure 5).

DISCUSSION

Point Mutations Establish the Importance of the Hydrogen Bond Network 3′ to the Cleavage Site for Specificity. In previous work, we compared the contacts that APE1 made to DNA to those made by a less specific nuclease, DNase I (*29*). Here we demonstrate that the contacts we identified as specific to APE1, a network of hydrogen bonds to the

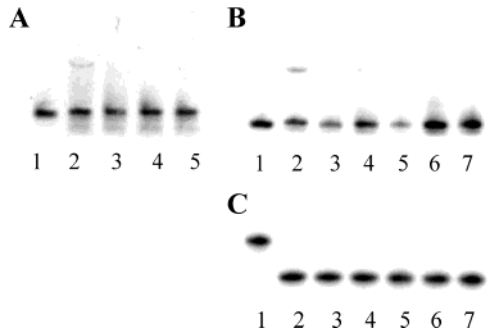

FIGURE 5: Cleaved DNA-binding by APE1 proteins. (A) 5′-Cy5 labeled 26-mer oligo containing a tetrahydrofuran (260 nM) was incubated with (1) no protein, or 20 ng of (2) the WT, (3) N226A, (4) N229A, (5) N226A/N229A in the presence of 2 mM EDTA and no divalent cation, and EMSA was carried out in nondenaturing gel electorophoresis. (B) 5′-^{32}P-labeled 43-mer oligo with a tetrahydrofuran was incubated with (1) no protein, or 20 ng of (2) the WT, (3) N226A, (4) N229A, (5) R177A, (6) R177A/N226A, and (7) N226A/N229A. (C) the samples in panel B were run in denaturing gel electrophoresis to separate the substrate and product DNA.

backbone of the DNA 3′ to the AP site, do indeed contribute to the affinity of the enzyme for both substrate and product (*28*, *37*). We created point mutations of mammalian APE1 that, based on a modular decomposition analysis of the sequences of APE family members (*29*), should disrupt binding to the phosphate groups 3′ of the AP site in damaged DNA. We showed that single mutations, which affect the interface between APE1 and the DNA backbone downstream of the AP site, increased the rate of cleavage of AP sites, which was probably due to enhanced turnover of the enzyme, compared to the WT protein. A double mutant that removed binding to two different 3′ phosphate groups greatly reduced activity, concomitant with a drastic drop in the ability of the mutant to bind either substrate or product DNA.

The role of R177, which penetrates the major groove of the AP site containing DNA in the cocrystal structure and forms a hydrogen bond to the DNA backbone one nucleotide downstream of the AP site (*28*, *38*), was previously examined for the overall reaction rate (*28*). It was concluded that the residue did not play a significant role in catalysis, because alanine substitution for the residue did not inactivate the protein, but enhanced its specific activity for the AP site-cleavage. The decomposition analysis of DNase I family members (*29*) revealed that in addition to R177, N226, and possibly N229 formed a network of H-bonds 3′ to the cleavage site on the damaged DNA. No such binding was seen in cocrystal structures of the nonspecific DNase I. This result led to the hypothesis that the network was essential for the specific cleavage of AP-sites by APE1. Further, we suspected that this network was also needed to enhance product binding after the cleavage reaction, which was seen in a cocrystal structure of APE1 (*28*, *37*).

As previously observed, R177A mutant had reduced affinity (higher K_m) for substrate DNA, while the V_{max} was enhanced. We hypothesized that release from the product inhibition must account for the enhanced rate of cleavage observed for the mutant. In this work, a double mutant of R177 with another residue with similar binding, N226, enabled us to determine the role of R177A in the substrate recognition more precisely. In one of the three cocrystal structure (1DE8), N229 interacts with the same phosphate as R177, and N226 interacts with the next phosphate downstream in other cocrystal structures (1DE9 and 1DEW). Both N226A and N229A had higher V_{max} values than the WT protein and also showed higher K_m values. Combining the N226A mutation with N229A greatly increased the K_m value for the APE reaction, and the ability to bind the damaged DNA was decreased drastically in the N226A/R177A double mutant (Figure 3). Furthermore, the specific activity of R177A/N226A double mutant was considerably decreased by almost 20-fold. These results show the additive effects of loss of the H-bonds, and demonstrate conclusively that bonds 3′ of the AP-site are necessary for specific DNA binding by APE1.

We can compare these results to those obtained for N212 (*39*), which is absolutely conserved in all DNase I family members regardless of substrate specificity. Alanine substitution of N212 completely inactivates the enzyme (*39*). Further, N212 makes a hydrogen bond to the scissile phosphate before the reaction that is not seen in crystal structures of the cleaved complex. Thus, while N212 is definitely necessary for the AP endonuclease activity of the enzyme, it does not contribute to the unique aspects of APE1 binding to damaged DNA and the cleaved product.

Sequence Decomposition Reveals the Important Segments for Specific Binding and Activity. The N226/N229 side chains were selected for point mutations based on a sequence decomposition of multiple sequence alignments of the APE family and subfamily coupled with a structural analysis (*29*). In that study, it was shown that other enzymatic families, with a common metal-based phosphorolytic cleavage but differing substrate choice, share five of the molegos. For example, in a complex of the bacterial inositol polyphosphate 5′-phosphatase (IPP) with inositol polyphosphate (IP), the geometry of the catalytic core residues and the substrate is remarkably similar to that seen in the APE1/DNA complex. The residues in IPP near the P−O bond cleavage site of inositol phosphate are very similar to those in APE near the scissile P−O of the AP-site (*28*, *40*). By eliminating these common areas that form a general metalophosphatase site, we could isolate those areas that are involved in general substrate binding. By further eliminating areas that bound undamaged DNA in the nonspecific nuclease DNase I, we could isolate the DNA binding residues that could most account for specific binding to damaged DNA.

The methodology used here provides a practical approach to design in vitro experiments to study the amino acid basis of specific protein functions. In the present study, we have shown that our approach is useful to produce variants of the APE1 with altered binding specificity and activity. The approach is not limited to missense mutations, but allows rigorous alteration of large segments (i.e., region replacement). In future work, we should be able to use this form of analysis to isolate areas that may be involved in the less studied aspects of APE activity, such as the redox based gene regulation.

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