The Exonuclease Activity of Human Apurinic/Apyrimidinic Endonuclease (APE1)

BIOCHEMICAL PROPERTIES AND INHIBITION BY THE NATURAL DINUCLEOTIDE Gp4G*

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Kai-ming Chou and Yung-chi Cheng‡

From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06520

Human DNA apurinic/apyrimidinic endonuclease (APE1) plays a key role in the DNA base excision repair process. In this study, we further characterized the exonuclease activity of APE1. The magnesium requirement and pH dependence of the exonuclease and endonuclease activities of APE1 are significantly different. APE1 showed a similar $K_m$ value for matched, 3’ mispaired, or nucleoside analog β-L-dioxolane-cytidine terminated nicked DNA as well as for DNA containing a tetrahydrofuran, an abasic site analog. The $K_m$ for exonuclease activity on matched, 3’ mispaired, and β-L-dioxolane-cytidine nicked DNA are 2.3, 61.2, and 98.8 min$^{-1}$, respectively, and 787.5 min$^{-1}$ for APE1 endonuclease. Site-directed APE1 mutant proteins (E96A, E96Q, D210E, D210N, and H309N), which target amino acid residues in the endonuclease active site, also showed significant decrease in exonuclease activity. Gp4G was the only potent inhibitor to compete against the substrates of endonuclease and exonuclease activities among all tested naturally occurring ribo-, deoxyribonucleoside/nucleotides, NAD$^+$, NADP$^+$, and Ap4A. The $K_i$ values of Gp4G for the endonuclease and exonuclease activities of APE1 are 10 ± 0.6 and 1 ± 0.2 μM, respectively. Given the relative concentrations of Gp4G, 3’ mispaired, and abasic DNA, Gp4G may play an important role in regulating APE1 activity in cells. The data presented here suggest that the APE1 exonuclease and AP endonuclease are two distinct activities. APE1 may exist in two different conformations, and each conformation has a preference for a substrate. The different conformations can be affected by MgCl$_2$ or salt concentrations.

DNA base excision repair is the main pathway to repair DNA base damages caused by oxidation, radiation, and the loss of bases (1, 2). There are several enzymes that participate in this pathway, including DNA polymerase β, DNA ligase III-XRCC1 complex (1, 2), and apurinic/apyrimidinic endonuclease (APE1) to protect the genome integrity (1–5). The DNA repair activity of APE1 resides in the C-terminal region (4, 5). It endonucleolytically makes a nick immediately adjacent to 5’ of an apurinic/apyrimidinic (AP) site and generates a hydroxyl group at the 3’ terminus upstream of the nick and a 5’-deoxyribose phosphate moiety downstream (6). DNA polymerase β further processes the product of APE1 by inserting a nucleotide into the gap and releasing the 5’-deoxyribose phosphate by its intrinsic lyase activity (7). The repair process is then completed by either DNA ligase I or DNA ligase II/XRCC1 to seal the nicked DNA (1). During DNA base excision repair, DNA polymerase β is the major polymerase that incorporates a nucleotide into the gapped DNA (1). However, DNA polymerase β is a lower fidelity polymerase (8) in comparison to the replicating DNA polymerases δ or ε (9). The error rate of polymerase β is about 1 in 4000 incorporations (1).

APE1 is a versatile multifunctional protein (10). In addition to its endonuclease activity, it also possesses 3’-phosphatase, 3’-phosphodiesterase, RNase H, and 3’–5’-exonuclease activities (10). APE1 knockout mice die in the early embryonic stage (11), which indicates this protein is critical for development. Previously we reported (12) that the 3’–5’-exonuclease activity of APE1 is the major exonuclease activity in the human cell nucleus for the removal of the nucleoside analog β-L-dioxolane-cytidine (L-OddC, BCH-4556, Troxacitabine, and Troxatyl) from the 3’ termini of DNA. L-OddC is a novel nucleoside analog with L-configuration that is currently under phase III clinical trial and is showing promising activity for the treatment of leukemia (13, 14). The incorporation of L-OddC into DNA terminates DNA chain elongation because of the lack of the 3’-hydroxyl group on the sugar moiety of L-OddC. The cytotoxicity of L-OddC is proportional to the amount of L-OddC in DNA, suggesting that the mechanism of action of L-OddC is to stop DNA replication. Therefore, the exonuclease activity of APE1 may play a critical role in determining the cytotoxicity of L-OddC (15). We also discovered that APE1 has a significant preference for the removal of mispaired nucleotides from the 3’ terminus of DNA when compared with matched pairs (16). Because physical interaction between polymerase β and APE1 had been established (17) and APE1 showed a significant preference for 3’ mispaired nicked DNA (16), APE1 could be the proofreading enzyme correcting the misincorporations introduced by DNA polymerase β (16).

In addition to its DNA repair activities, APE1 was shown to possess redox activity, which regulates the DNA binding of a number of transcription factors, including Jun and Fos (18, 19). The redox activity of APE1 resides in the N-terminal region (5), and cysteine 65 is considered to be the active site (4, 20). Previous reports (4, 20) indicated that Cys-65 has no impact on the DNA endonuclease activity of APE1. The biological function of this redox activity of APE1 is, however, not well understood.

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, Yale University School of Medicine, 333 Cedar St., SHM B315, New Haven, CT 06520. Tel.: 203-785-7119; Fax: 203-785-7129; E-mail: cheng.lab@yale.edu.

§ The abbreviations used are: APE1, human apurinic/apyrimidinic endonuclease; L-OddC, β-L-dioxolane cytidine; Gp4G, PpP-di(guanosine-5’)-tetrathosphate; Ap4A, PpP-di(adenosine-5’)-tetrathosphate; F-DNA, DNA containing a tetrahydrofuran; AP, apurinic/apyrimidinic.
Several groups (21–23) have solved the crystal structure of APE1 in the presence or absence of abasic DNA. Mol et al. (22) reported the crystal structure of APE1–AP DNA complex and proposed a mechanism of action for the APE1 endonuclease activity in which amino acid Glu-96 binds to a metal ion, His-309/Asp-283 interacts with the phosphate backbone of DNA, and Asp-210 deprotonates a water molecule during nucleophilic attack on the AP site (22). Beernink et al. (23) subsequently proposed another mechanism of action based on the structure of APE1 crystallized under a neutral pH in the absence of AP DNA. According to the latter model, there are two metal-binding sites in the active center of APE1 endonuclease; Glu-96 is involved in coordinating one metal ion, which stabilizes the O-3’ leaving group (23); and Asp-210 and His-309 coordinate the other metal ion to deprotonate a water molecule, which generates a nucleophile. In both models, Glu-96, Asp-210, and His-309 are involved in endonucleolytic activity of APE1. Mutations at these sites resulted in a significant decrease in endonuclease activity.

The monophosphate metabolites of ribonucleosides and several nucleoside analogs have been shown to have inhibitory effects on DNA exonucleases and DNA polymerase-associated exonucleases (24–27). In this study, we have characterized the biochemical properties of APE1 including optimal pH, $\text{Mg}^{2+}$ requirements, and kinetic parameters of matched, 3’ mispaired as well as 3’-OddC terminated DNA. The role of the specific amino acids Cys-65, Glu-96, Asp-210, and His-309 in the exonucleolytic action of APE1 was also examined. In addition, we have examined the potential inhibitory effects of naturally occurring ribonucleosides and deoxyribonucleosides and nucleotides on both exonuclease and endonuclease activities of APE1. Interestingly, among all the nucleosides and nucleotides, the dinucleotide compound GpG was the only potent inhibitor of both endonuclease and exonuclease activities of APE1. GpG is a product of GTP:GTP guanylyltransferase (28, 29) or originates from the low activity of mRNA capping enzymes. The sequences of the 3’-end-labeled with $^32P$-end-labeled with $[\text{C}]$ were obtained under pH 6.2 and 7.5 (22, 23), which suggested that pH could cause conformational changes in APE1. We therefore determined the optimal pH for both the endo- and exonuclease activities of APE1 under otherwise identical reaction conditions. In addition, DNA substrates used shared the same sequence context to eliminate the potential influence of sequence context (see “Experimental Procedures”). As shown in Fig. 1a, optimal exonuclease activity was detected at pH 7.4 using a 3’-mispaired nicked DNA as substrate, and the

**EXPERIMENTAL PROCEDURES**

**Materials and Compounds—**$[^{32}]P$ATP and terminal deoxynucleotidyldtransferase were purchased from Amersham Biosciences. 3-OddCCTP was synthesized in our laboratory as described (31). T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). GpG was purchased from Sigma, and purity (95%) was confirmed by high performance liquid chromatography.

**Oligonucleotide Substrates—**All oligonucleotides were synthesized by Integrated DNA Technology, Inc. (Coralville, IA), and further purified by electrophoresis on a 20% denaturing polyacrylamide gel. The 21-mer oligonucleotides were 5’-end-labeled with $[^{32}]P$ATP using T4 polynucleotide kinase. The 5’-labeled 21-mer was then annealed to a 40-mer oligonucleotide to generate a recessed DNA with or without a mismatch at the 3’ terminus. To generate nicked mismatched and mismatched DNA, additional 19-mer oligonucleotide was added for the annealing reaction. The sequences of the 3’-mispaired recessed (Sequence 1) or nicked DNA (Sequence 2) are shown.

$[^{32}]P$ATG GCG CGG AGA CTG GAA ATT TGC GCC GGG GAA TTC C
CAC CCC GCC TCT GAA TCT CTG TAA ACC GCG CCC CTT AAG C

**Sequence 1**

$[^{32}]P$ATG GCG CGG AGA CTG GAA ATT TGG GCC GGG GAA TTC C
CAC CCC GCC TCT GAA TCT CTG TAA ACC GCG CCC CTT AAG C

**Sequence 2**

$[^{32}]P$ATG GCG CGG AGA CTG GAA ATT TGG GCC GGG GAA TTC C
CAC CCC GCC TCT GAA TCT CTG TAA ACC GCG CCC CTT AAG C

**Sequence 3**

The sequence for the AP site analog (tetrahydrofuran, F) DNA (F-DNA) is shown in Sequence 3.

**Site-directed Mutagenesis—**The pET-28b expression plasmid containing wild type APE1 was a generous gift from Dr. Bruce Demple (Harvard University). Mutagenesis was performed by the PCR-based method using the QuickChange Site-directed Mutagenesis Kit (Stratagene). The primers used to create each mutant form are as follows: APE1-C65A primer, 5’-GCC ACA CTC AAG ATC GCC TCT TGT GAT GAT GCG; APE1-E96A primer, 5’-ATA CTG TGC CTT CAA GCG ACC AAA TGT TCA GAG AAC; APE1-E96Q primer, 5’-ATA CTG TGC CTT CAA GAC GGG ACC AAA TGT TCA GAG AAC; APE1-D210E primer, 5’-CTT GTG CGT TGT GGA GAG CTC AAT GTG CGA CAT GAA; APE1-H309N primer, 5’-GCC CTC GCC GGT CAT GAG TAC CCT GCT ATC ACC CTA TAC.

The DNA sequences of the expression plasmids containing the wild type or the site-directed mutant APE1 genes were confirmed by DNA sequencing at HHMI/Keck Facility, Yale University.

**Purification of Recombinant APE1 and the Site-specific Mutant APE1 Proteins—**The wild type and mutant pET-28b-APE1 constructs were expressed in Escherichia coli BL21(DE3) cells. The cells were grown at 37 °C in a shaking incubator until the culture reached an $A_{600}$ of 0.6. Expression of APE1 from the T7 promoter was induced for 3 h by addition of 1 mM of isopropyl-$\beta$-d-thiogalactopyranoside (final concentration). The cells were then harvested and lysed by sonication, and the cell debris was pelleted by ultracentrifugation (27,500 rpm, 4 °C, 40 min in a Beckman Ti 80 rotor). The supernatant was dialyzed against nickel column binding buffer (Buffer A: 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 20 mM imidazole) before loading onto a Ni$^{2+}$-charged His-trap fast protein liquid chromatography column (Amersham Biosciences), and the bound protein was eluted with a 20–300 mM imidazole gradient in binding buffer. The elution fractions that contained APE1 protein were pooled and dialyzed against Buffer B (20 mM Tris-HCl, pH 7.4, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA, and 20 mM NaCl). The dialyzed sample was then applied to a 1-ml fast protein liquid chromatography Hitrap SP XL column (Amersham Biosciences). The bound protein was eluted with a linear gradient of 100% Buffer A to 100% Buffer B (20 mM Tris-HCl, pH 7.4, 10% glycerol, 1 mM EDTA, and 1 mM dithiothreitol, 1 x NaCl).

The purified proteins were stored at −70 °C in storage buffer (20% glycerol, 20 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA). All proteins were >95% pure as judged by electrophoresis on 12% SDS-polyacrylamide gels stained with Coomassie Blue (data not shown).

**Enzyme Assays—**Standard reaction (10 µl) mixtures contained 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mg/ml bovine serum albumin, optimal MgCl$_2$, and various concentrations of DNA substrates and enzymes. The reaction mixtures were incubated at 37 °C for various times and then stopped by adding 4 µl of gel loading solution (90% formamide, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue) and heated at 80 °C for 3 min. Samples (4 µl) were loaded onto a 20% denaturing polyacrylamide gel for electrophoresis. The gel was then dried under vacuum and subjected to autoradiography and a PhosphorImaging screen (Bio-Rad). For kinetic analysis, the concentration of DNA substrates ranged from 1 to 500 nM at a fixed enzyme concentration. For each reaction, less than 25% of the substrates were converted to products by the enzyme, even at the lowest substrate concentration. All the kinetic data were determined based on the initial reaction rates.

**RESULTS**

**The Optimal pH for the Exonuclease and Endonuclease Activities of APE1—**Two different crystal structures of APE1 were obtained under pH 6.2 and 7.5 (22, 23), which suggested that pH could cause conformational changes in APE1. We therefore determined the optimal pH for both the endo- and exonuclease activities of APE1 under otherwise identical reaction conditions. In addition, DNA substrates used shared the same length and sequence context to eliminate the potential influence of sequence context (see "Experimental Procedures"). As shown in Fig. 1a, optimal exonuclease activity was detected at pH 7.4 using a 3’-mispaired nicked DNA as substrate, and the
Studies of APE1 Exonuclease on 3’ Mispaired DNA

Magnesium Requirement for the Endonuclease and Exonuclease Activities of APE1—Magnesium is required for the endonuclease activity of APE1, but high concentrations of magnesium inhibit this activity (32, 33). We have demonstrated previously (12) that magnesium is also required for the exonuclease activity of APE1. The recent crystal structure of APE1 revealed that there are two divalent metal ion-binding sites in the active center at neutral pH (7.5) (23), but only one divalent metal ion was observed in the crystal structure of the APE-DNA complex at pH 6.5 (22). This suggested that optimal Mg2+ concentrations for the endonuclease activity might vary under different assay conditions, e.g., pH. We therefore determined the optimal Mg2+ concentration required for the exonucleolytic and endonucleolytic activities at the same pH (7.4).

As shown in Fig. 1b, neither activity was observed in the absence of magnesium. The maximal exonuclease activity was observed between 0.1 and 1.0 mM MgCl2 on a 3’ mispaired recessed DNA substrate. Interestingly, a higher concentration of MgCl2 was required to reach the maximal exonuclease activity for the removal of 3’ mispair from a nicked DNA substrate. Similar Mg2+ dependence profiles were observed while using recessed or nicked DNA with l-oddC at the 3’ terminus (data not shown). In contrast to the exonuclease activity, the endonuclease activity required a much higher magnesium concentration to reach its optimal activity (10–15 mM) under the same assay conditions.

The higher magnesium requirement for optimal endonuclease activity compared with the exonuclease activity may be due to a need for higher ionic strength in the former reaction. In other words, both activities require a similarly low Mg2+ concentration (around 2 mM) for chemical catalysis, and the additional contribution of the metal (around 15 mM) to the endonuclease activity is to increase the ionic strength of the solution. To address this issue, both activities were monitored at 2 mM MgCl2 with increasing amounts of NaCl using either 3’ mispaired nicked DNA or F-DNA as substrates. As shown in Fig. 1c, similar exonuclease activity was observed with 0–60 mM NaCl, but this activity decreased significantly above 100 mM. In contrast, the endonuclease activity increased in a salt-dependent manner. The maximal endonuclease activity was observed at 120 mM NaCl, the same activity as observed using 12.5 mM MgCl2 and 20 mM NaCl. The endonuclease activity decreased dramatically when the NaCl concentration exceeded 300 mM.

Under a reaction mixture containing 12.5 mM MgCl2, only an inhibitory effect was observed with increasing concentrations of NaCl (data not shown). Thus, the higher MgCl2 concentration for optimal endonuclease activity did not appear to be a specific requirement during endonucleolytic catalysis.

The Steady-state Kinetic Studies of APE1 Exonuclease—Because the magnesium and salt requirements for endonuclease and exonuclease activities are different, the kinetic parameters of both activities were studied under the optimal conditions for each activity of APE1. To determine the $k_{cat}$ and $K_m$ values, the exonuclease activity of APE1 was measured using varied concentrations of MgCl2 at the test pH, 7.4, and varied concentrations of NaCl. The reaction mixtures were incubated at 37°C for 5 min. This figure is representative of three independent experiments.
Studies of APE1 Exonuclease on 3' Mispaired DNA

For exonuclease activity, the buffer contained 20 mM Tris-HCl, pH 7.4, 12.5 mM MgCl₂, 20 mM NaCl, and 0.1 mg/ml bovine serum albumin. For the exo-nuclease activity on recessed DNA and nicked DNA, the same buffer was used but the MgCl₂ concentrations were 0.1 and 2 mM, respectively. The enzyme concentration used for the exo-nuclease activity on 3' matched nicked DNA substrate was 100 μM. The enzyme concentration for 3' mispaired or L-OddC terminated recessed or nicked DNA was 11.1 μM. The enzyme concentration for the endo-nuclease activity on F-DNA was 1.2 μM.

| Nicked DNA          | $K_m$ (μM) | $k_{cat}$ (min⁻¹) |
|---------------------|------------|-------------------|
| Match               | 16.2 ± 2.8 | 2.3 ± 0.1         |
| Mismatch            | 12.6 ± 1.3 | 61.2 ± 1.9        |
| 3' L-OddC           | 19.8 ± 2.5 | 98.8 ± 4.7        |

| Recessed DNA        | $K_m$ (μM) | $k_{cat}$ (min⁻¹) |
|---------------------|------------|-------------------|
| Mismatch            | 37.8 ± 3.6 | 63.0 ± 2.8        |
| 3' L-OddC           | 41.3 ± 6.2 | 136 ± 11.2        |
| F-DNA               | 10.7 ± 1.5 | 787.5 ± 33.8      |

Table I: The kinetic parameter of APE1 DNA endonuclease and exonuclease

The specific enzyme activities of wild type or mutant APE1 proteins were determined by the initial reaction rates of their endonuclease and exonuclease activities in the presence of 50 nM F-DNA, mispaired recessed or nicked DNA under their optimal reaction conditions (same as Table I). The concentrations of wild type and C65A mutant APE1 proteins used are the same as Table I. For E96A mutant APE1, 10 and 1.1 nM protein were used in the exonuclease and endonuclease assays, respectively. For E96Q mutant APE1, 10 nM enzyme was used for the endonuclease assay, and 3.3 and 1 nM protein were used for the 3' mispaired recessed and nicked DNA substrates, respectively. A final concentration of 10 nM enzyme was used for the endonuclease and exonuclease activity assays for D210E, D210N, and H309N mutant APE1.

| Recessed DNA | Nicked DNA | F-DNA |
|--------------|------------|-------|
| WT           | 3 × 10⁶    | 1.4 × 10³ | 2.2 × 10⁴ |
| C65A         | 3.6 × 10⁶  | 2.5 × 10³ | 1.3 × 10⁴ |
| E96A         | 0.03       | 0.23   | 0.21   |
| E96Q         | 1.2        | 0.21   |       |
| D210E        | 0.02       | 0.46   | 0.2    |
| D210N        | ND         | ND     | ND     |
| H309N        | ND         | ND     | 0.04   |

ND, not detectable.

The Roles of Specific Amino Acids Involved in the Exonuclease Activity—The different magnesium requirements, salt dependence, and kinetic parameters for the endonuclease and exonuclease activities of APE1 suggested that amino acids involved in these two activities may play different roles during the reactions. To examine whether the exonuclease activity of APE1 shares the same amino acids in the active center with endonuclease activity, specific site-directed APE1 mutants of amino acids Glu-96, Asp-210, and His-309, which have been shown to be critical for the endonuclease activity (10, 33) were generated. The mutants were designed to probe the impact of both the electrostatic charge and spatial effects of these amino acid residues (see Table II). Cys-65, a key player in the redox activity of APE1 (4, 20) was also mutated to examine its potential involvement in the exo-nuclease activity of APE1. The specific activities were obtained from the initial reaction rate of the wild type, and mutant APE1 proteins on recessed and nicked DNA with a 3' mispair (sequences are shown under “Experimental Procedures”) as well as F-DNA are presented in Table II. The endo-nuclease activity of wild type APE1 is about 30-fold higher than the exo-nuclease activity on 3' mispair from a nicked DNA and 80-fold higher than from the recessed DNA. Both endonuclease and exo-nuclease activities of Cys-65 mutant APE1 were similar to those of the wild type, which suggested that Cys-65 is not involved in either activity. Glu-96 was shown to be important for the magnesium coordination during APE1 endonuclease action (23). As shown in Table II, the endo-nuclease activity of E96A decreased 10⁴-fold as compared with the wild type APE1. The exonuclease activity of E96A decreased about 10⁴-fold on 3' mispaired recessed DNA and 6,100-fold on nicked DNA. E96Q showed better (35-fold) exonuclease activity on both recessed and 3' mispaired DNA than E96A. The Mg²⁺ dependence profiles of E96Q exonuclease activity were very similar to that of wild type, but the endo-nuclease activity of E96Q showed no significant change under different Mg²⁺ concentrations, although Mg²⁺ was required (Fig. 2a). Moreover, the product of E96Q endonuclease activity only increased slightly with time (Fig. 2b), which is not the pattern observed with wild type or other APE1 mutant proteins. To explore the interesting results of E96Q exonuclease activity, we performed a mobility retardation assay in the presence or absence of magnesium (Fig. 2c). The results showed that magnesium increased the formation of E96Q-F-DNA complex (Fig. 2c, lane 5). Under the same conditions, no complexes formed using wild type (Fig. 2c, lane 4) or other mutant APE1 proteins (data not shown), which suggested that dissociation of E96Q from its exonuclease product is very slow.

D210E mutant APE1 protein showed similar reduction in both endonuclease and exonuclease activities as the E96A mutant APE1 protein. D210N APE1 mutant protein also showed very low endonuclease and exonuclease activities. The H309N mutant APE1 showed no detectable exonuclease activity, and the endonuclease activity decreased more than 10⁵-fold in comparison to the wild type. The double mutant E96A/D210A showed no endonuclease and exonuclease activities, which suggested that there is no compensatory effect between these two amino acids (data not shown).

With the exception of E96Q, the magnesium requirement profiles of C65A, E96A, and D210A were similar to those of the endonuclease or exonuclease activities of the wild type APE1 (data not shown).

Inhibition of Endonuclease and Exonuclease Activities of APE1 by Dinucleotide GpG—The monophosphate metabolites of purine ribonucleosides and some nucleoside analogs were shown to inhibit DNA exonuclease activities (24–27). In this study, the naturally occurring ribo- and deoxyribonucleotides, dinucleo-
The inhibitory effects of nucleoside and nucleotides on the endonuclease and exonuclease activity of APE1

| Nucleosides/nucleotides | Exonuclease activity % | Endonuclease activity % |
|-------------------------|------------------------|------------------------|
| NMP                     | <5                     | <5                     |
| dNMP                    | <5                     | <5                     |
| NDP                     | <5                     | <5                     |
| dNDP                    | <5                     | <5                     |
| NTP                     | <5                     | <5                     |
| dNTP                    | <5                     | <5                     |
| NADH                    | <5                     | <5                     |
| NADPH                   | <5                     | <5                     |
| ApA                     | <5                     | <5                     |
| GpG                     | >95                    | >95                    |

APE1 exists as a monomer in the cell (10), and there is only one active site found within in this enzyme (22, 23). Therefore, the $K_i$ values of a competitive inhibitor on both endonuclease and exonuclease should theoretically be the same. Because the optimal concentrations of MgCl$_2$ for endonuclease and exonuclease activities are different, it is possible that MgCl$_2$ may affect the $K_m$ and $K_i$ values. To address this issue, the $K_m$ and $K_i$ values of APE1 endonuclease activity were determined under the optimal MgCl$_2$ concentration for APE1 exonuclease activity (2 mM). The results indicated that both the $K_m$ and $K_i$ values of endonuclease activity under this condition were similar to those under higher MgCl$_2$ concentration (12.5 mM) (data not shown) but $k_{cat}$ decreased, suggesting that MgCl$_2$ has no significant impact on $K_m$ and $K_i$ values. In addition, the $K_m$ value of APE1 exonuclease at 12.5 mM MgCl$_2$ did not show a significant change (~2-fold). The $K_i$ value of GpG on exonuclease, under 12.5 mM MgCl$_2$, could not be obtained as the long incubation time caused non-enzymatic breakdown of GpG.

**DISCUSSION**

We demonstrated previously (12) that APE1 is the major exonuclease that removes the unnatural 1-configuration nucleoside analog, l-OddC, as well as other l-nucleoside analogs from DNA. We subsequently discovered that the exonuclease activity of APE1 has a significant preference for the removal of 3' mispaired nucleotides from DNA over the matched ones (16). Because physical interaction between APE1 and DNA polymerase β has been established, this novel 3' mispair removal function of APE1 could explain why the mutation rate is lower than expected, given that the error rate of DNA polymerase β is 1 per 4000, and at least 10$^4$ abasic sites are generated per cell per day (1).

In this report, we have further characterized the biochemical properties of the exonuclease activity of APE1. Several crystal structures of APE1 have been solved in the presence or absence of abasic DNA under different crystallization conditions (21–23). Based on the structures, different mechanisms of action were proposed for the endonuclease activity of APE1. The latest crystal structure solved by Beernink et al. (23) indicated that there are two divalent ion binding sites in the APE1 active center under neutral pH. Given the fact that the optimal pH for APE1 endonuclease activity is between 6.6 and 8.6 (23), the
The reaction conditions are the same as described in the previous report (23).

Both endonuclease and exonuclease activities require magnesium for the catalytic reactions (12, 33). At pH 7.5, the optimal magnesium requirement for exonuclease activity (0.1–2 mM) is substantially lower than that of endonuclease (10–15 mM). Interestingly, as shown in Fig. 1c, at a low MgCl₂ concentration (2 mM) NaCl could achieve the same endonuclease activity as the higher concentration of MgCl₂ (12.5 mM). This suggests that both endonuclease and exonuclease activities of APE1 may have the same MgCl₂ requirement; however, the higher MgCl₂ concentration requirement for the optimal endonuclease activity may not be specific because NaCl can achieve the same activity. The crystal structure showed that the abasic DNA was severely distorted from B-form DNA during the endonuclease action (22). Although the $K_m$ values of APE1 exonuclease on 3’ mispaired nicked DNA and endonuclease on F-DNA are very similar, despite different MgCl₂ concentrations (2 and 12.5 mM, respectively), the structure of nicked DNA is very different from AP-DNA (no strand breakage). Therefore, the higher concentrations of MgCl₂ or NaCl may facilitate the enzyme conformation changes to favor its action on F-DNA.

The $K_m$ of APE1 exonuclease on recessed DNA is about 3-fold higher than on the nicked or F-DNA, which suggested that APE1 could have higher affinity for nicked or F-DNA than the recessed DNA. It also explains the higher efficiency of APE1 in the removal of 3’ mispaired nucleotide from nicked than from the recessed DNA (16), although the $k_cat$ values are similar for both substrates. In contrast, the $k_cat$ value of exonuclease on the matched DNA is significantly lower than for either 3’ mispaired or L-OddC DNA. These data further support our previous results (16) that APE1 has a higher efficiency for the removal of 3’ mispaired nucleotides than matched nucleotides from DNA. DNAs with L-OddC at the 3’ terminus were better substrates for the APE1 exonuclease activity as compared with the 3’ mispaired DNAs, because the $k_cat$ values are higher than those for the 3’ mispaired DNA on both recessed and nicked DNA. The unnatural L-configuration of L-OddC may cause a distortion in DNA rendering it a more favorable substrate. Under the optimal reaction conditions, F-DNA is the most favorable substrate for APE1, and the $k_cat$ value of endonuclease activity on the 3’ mispaired or L-OddC terminated nicked DNA, incubated at 37 °C for 5 min. For endonuclease activity, reaction conditions are the same as in the presence of various concentrations of GpG (lane 9, control DNA; lanes 10–16, 0, 1, 3, 10, 20, 30, and 100 μM, respectively). The results were analyzed by denaturing gel and exposed to a PhosphorImager screen for quantitation. The $IC_{50}$ is the concentration of GpG required to inhibit 50% of the reaction. c, GpG competitively inhibits exonuclease activity of APE1. The reaction condition is the same as described in Table 1 in addition to various concentrations of GpG (0–2 μM). The $K_m$ value (1 ± 0.2 μM) was determined by the $K_m^{obs}$ under different concentrations of GpG. d, GpG competitively inhibits APE1 endonuclease activity. The reaction conditions are the same as described in Table 1 in addition to various concentrations of GpG (0–30 μM). The $K_m$ value (10 ± 0.6 μM) was determined by the $K_m^{obs}$ under different concentrations of GpG. This figure is representative of three independent experiments.
respectively. More detailed pre-steady-state kinetic parameters are being explored to further the understanding of the interactions between APE1 and various DNA substrates as well as the catalysis of endonuclease and exonuclease cleavage.

The crystal structure of APE1 revealed that there is only one active center in APE1 (21–23), although different mechanisms of action for the endonuclease have been proposed. The redox activity of APE1 resides within the N-terminal region, and Cys-65 has been implicated to be the active center (4, 5, 19). The site-directed Cys-65 mutant APE1 protein was shown to possess similar endonuclease activity as wild type (4, 20). In this report, we further showed that C65A mutant APE1 has similar exonuclease activity as the wild type APE1 on both recessed and nicked 3’ mispaired DNA. These data further support that Cys-65 is not involved in the DNA repair activity of APE1.

All the crystal structures of APE1, obtained under various crystallization conditions, indicated that amino acids Glu-96, Asp-210, and His-509 were critical for the endonuclease activity (21–23). The site-directed mutagenesis results shown in Table II indicated that mutation of Glu-96 to alanine reduced both exonuclease and endonuclease activities of APE1 significantly (104 and 105, respectively), which suggested that Glu-96 was critical but might have a different impact on the two enzyme activities. The exonuclease activity of E96Q decreased about 230-fold as compared with that of the wild type APE1 but was ~35-fold higher than that of E96A, which indicated that the carboxyl group of Glu-96 might be critical for both enzyme activities. Moreover, E96Q exonuclease activity did not require higher MgCl2 concentration. Mobility shift studies using native gel electrophoresis showed the E96Q mutant but not the wild type or other mutant APE1 proteins bound to F-DNA in the presence of magnesium (12.5 mM). To explore whether E96Q APE1 and F-DNA formed covalent bonds, the complexes were boiled in the presence of SDS and electrophoresed on an SDS-PAGE gel. No complexes were observed after electrophoresis (data not shown), which indicated that no covalent bonds formed in the complex. These results suggested that the dissociation of E96Q mutant from its AP endonuclease product is very slow, and Glu-96 is critical in determining the $k_{cat}$ value for APE1 endonuclease activity. Glu-96 was proposed to play important roles in magnesium placement, and the lack of dependence on magnesium and the slow dissociation of E96Q from its DNA product suggested that the higher ionic strength requirement for the optimal APE1 endonuclease activity is needed for the enzyme to disassociate from its product.

The APE1 D210E mutant protein also showed a significant decrease in both endonuclease and exonuclease activities, although the substituted glutamic acid can provide the same carboxyl group as the aspartic acid. This indicated that steric hindrance has a profound effect on the enzyme reaction. The D210N mutant APE1 showed extremely low exonuclease and endonuclease activities, which supported the critical role of carboxyl group of glutamic acid (35). The H309N mutant showed very low exonuclease and endonuclease activities. Interestingly, the Glu-96 and Asp-210 APE1 mutant proteins, with the exception of E96Q, showed similar magnesium requirement profiles for endonuclease and exonuclease activities as the wild type APE1 on 3’ mispaired recessed DNA, nicked DNA, and F-DNA. We conclude that all the amino acids studied in this report are important for both endonuclease and exonuclease activities of APE1, but their roles in the two reactions may not be identical.

AMP and GMP were shown to be inhibitors for a number of DNA polymerase-associated exonuclease activities (24–27, 36). Here we report that the APE1 endonuclease and exonuclease activities cannot be inhibited by AMP, GMP, and other natural nucleoside or their mono-, di-, and triphosphate metabolites. Interestingly, Gp4G but not Ap4A competitively inhibited both activities of APE1. The intracellular concentration of Gp4G in mammalian cells is not clear; however, the physiological concentrations of Gp4G was shown to be in the nanomolar to micromolar range in rat tissues and human blood platelets (40, 41). In addition, the concentrations of Gp4G were in the nanomolar to micromolar ranges in Saccharomyces cerevisiae and E. coli cells, depending on the growth condition (37). In exponentially growing S. cerevisiae cells, the Gp4G concentration was determined to be 60 nM, whereas after the temperature shift to a non-growing condition, the concentration of Gp4G increased to 1.15 $\mu$M (37). This implies that the concentration of Gp4G may be higher in nonproliferating tissues in mammals. In Artemia salina, Gp4G comprises as high as 50% of the total nucleotide pool (10 mM) in dormant cysts, which was suggested to serve as a source of purine for DNA synthesis (38). It was also proposed that Gp4G regulates the activity of endoribonuclease VI in Artemia, by alternating its concentration under different developmental stages (39). An in vitro experiment has shown that Gp4G activates GMP reductase activity in both Artemia and human erythrocytes at nanomolar concentrations (41, 42). Grau et al. (43) proposed a relationship between Gp4G binding capacity and the time of development of rat embryos. It was also suggested that Gp4G might play a role in the regulation of organ perfusion and vascular growth (40).

Interestingly, Gp4G can also be used as a primer for template-directed synthesis of virus-specific oligonucleotides and RNA (30, 44). Furthermore, Gp4G can be synthesized by some viral mRNA guanylyltransferases when RNA capping is uncoupled from methylation and elongation (30). Several carbocyclic and 2’-deoxy analogs of Gp4G were shown to be substrates for human immunodeficiency virus-reverse transcriptase (30, 31), which suggested that Gp4G may also affect viral replication. Given the specificity and potency reported in this study and potential different concentrations of Gp4G in different intracellular compartments as suggested by others (38, 39), it is conceivable that Gp4G may play important roles in regulating the enzyme activities of APE1 in the cell nucleus.

In summary, this report reveals that APE1, in addition to its most preferable endonuclease activity on apurinic sites, also has very good activity on 3’ mispaired DNA and DNA terminated with the unnatural 1-configuration nucleoside analogs. Both endonuclease and exonuclease activities of APE1 may have similar active centers, but the amino acids involved may play different roles during each reaction. MgCl2 is required for both activities, but higher ionic strength is required for endonuclease activity. It is possible that higher ionic strength induces a change to a endonuclease-favored conformation. The different $K_i$ values of Gp4G on the two activities further supported the hypothesis that APE1 may have two different conformations in equilibrium, and the conformations can be affected by higher concentrations of MgCl2 or NaCl. Gp4G may have different affinity to these two forms; one form has a preference for the exonuclease activity (lower ionic strength), and the other form favors endonuclease activity (higher salt and magnesium requirement). We are currently solving the co-crystal structures of APE1 with 3’ mispaired nicked DNA under different conditions to address this question.

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