Capturing snapshots of APE1 processing DNA damage

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DNA apurinic-apyrimidinic (AP) sites are prevalent noncoding threats to genomic stability and are processed by AP endonuclease 1 (APE1). APE1 incises the AP-site phosphodiester backbone, generating a DNA-repair intermediate that is potentially cytotoxic. The molecular events of the incision reaction remain elusive, owing in part to limited structural information. We report multiple high-resolution human APE1–DNA structures that divulge new features of the APE1 reaction, including the metal-binding site, the nucleophile and the arginine clamps that mediate product release. We also report APE1–DNA structures with a T-G mismatch 5′ to the AP site, representing a clustered lesion occurring in methylated CpG dinucleotides. These structures reveal that APE1 molds the T-G mismatch into a unique Watson-Crick-like geometry that distorts the active site, thus reducing incision. These snapshots provide mechanistic clarity for APE1 while affording a rational framework to manipulate biological responses to DNA damage.

RESULTS

Previous DNA-bound APE1 substrate and product structures have been determined to 2.95 and 2.65 Å, respectively, and have provided clues as to the mechanism of APE1 (ref. 11). Yet, the resolution of these structures has allowed limited understanding of the molecular features of AP-site recognition and processing. To resolve mechanistic uncertainties, we determined several high-resolution structures of APE1 substrate and product complexes.

High-resolution structure of APE1 bound to product DNA

We obtained the APE1 product complex in the presence of MgCl₂ with a 21-mer double-stranded DNA containing a centrally located AP-site analog (tetrahydrofuran, THF). The crystals diffracted to 1.57 Å (Table 1) and revealed APE1 flipping the AP site out of the double helix and into the active site binding pocket and kinking the DNA by ~35°. This configuration places the AP site in position for incision of the 5′-phosphate backbone and results in an orphan base opposite the AP site (Fig. 1a). Overall, the protein and DNA structure is globally consistent with the previously reported 2.4-Å product structure18 (r.m.s. deviation of 0.29 Å over 316 Cα atoms; Supplementary Fig. 1).

A detailed view of the active site shows a single Mg²⁺ (Fig. 1b,c). The Mg²⁺ coordinates E96 and three water molecules that are also coordinated to D70 and D308. Mutation of these aspartate residues has been shown to reduce activity23,24. Importantly, the Mg²⁺ in this product complex directly coordinates the oxygen of the 5′-phosphate and 3′-hydroxyl at the site of backbone cleavage, ligands that are generated after cleavage. The nonbridging oxygens of the 5′-phosphate interact with N212, D210, Y171 and H309 (Fig. 1c). The bridging oxygen of the cleaved AP site is within hydrogen-bonding distance to N174. Together, these residues stabilize the cleaved product complex.

Product binding facilitated by swinging of arginine clamps

The pre–steady state kinetic description of APE1 is that rapid catalysis is followed by slow product release25. This feature may conceal cytotoxic incised DNA BER intermediates during DNA-damage
processing. In our product structure, we identified a unique contact with R181 not appreciated previously. R181 has been observed to come within hydrogen-bonding distance of E154 in the DNA-bound enzyme and apoenzyme APE1 structures 11,26. In our product complex, R181 shifts 5.5 Å to come within hydrogen-bonding distance of a backbone phosphate upstream (5′) of the AP site (Fig. 2a). In this conformer, side chain nitrogens of R181 are 2.7 and 2.8 Å away from a DNA-backbone nonbridging oxygen. These contacts are present only after product formation, as shown by an overlay with the apoenzyme and substrate complexes (Fig. 2a), thus implying that R181 facilitates product binding by shifting to clamp down on product DNA after catalysis.

To probe the role of R181, we performed protein-DNA binding studies, using full-length wild-type or R181A mutant proteins and substrate or product DNA. We included hairpin oligonucleotides in the DNA to prevent end binding (Fig. 2b) and performed the substrate-DNA binding experiments with fluorescence anisotropy in the presence of 10 mM EDTA to prevent catalysis. Both wild-type and R181A APE1 exhibited a low-affinity nonspecific binding component and a high-affinity binding component. For the high-affinity component, both proteins bound to the substrate DNA with similar subnanomolar affinity (K_d = 0.4 nM).

In contrast, binding analysis with product DNA indicated the R181A mutant enzyme bound with approximately three-fold-weaker affinity than that of the wild-type enzyme (Fig. 2b).

### Table 1 Data collection and refinement statistics

| Data collection | Product complex | 2′-OMe-PS substrate complex | 2′-OMe-PS substrate complex (MnCl_2) | E96Q D210N mismatch substrate | Mismatch product |
|----------------|-----------------|-----------------------------|-------------------------------------|-----------------------------|----------------|
| Space group    | P1              | P1                          | P1                                 | P1                          | P1             |
| Cell dimensions|                 |                             |                                     |                             |                |
| a, b, c (Å)    | 44.5, 61.7, 72.1| 44.4, 60.8, 73.2            | 44.3, 60.6, 73.3                   | 44.5, 60.7, 73.2            | 44.4, 61.6, 72.3|
| α, β, γ (°)    | 83.9, 78.8, 88  | 83.0, 80.5, 89.1            | 83.1, 80.6, 89.0                   | 82.8, 80.3, 89.2            | 83.8, 78.7, 88.2|
| Resolution (Å) | 50–1.57         | 50–1.63                     | 50–1.80                            | 50–1.85                     | 50–1.95        |
| Rmerge (%)     | 11.5 (57.4)     | 8.4 (54.1)                  | 6.9 (56.1)                         | 13.2 (43.0)                 | 5.8 (38.0)     |
| Completeness (%)| 96.6 (94.8)     | 98.7 (88.3)                 | 99.8 (99.2)                        | 100 (99.9)                  | 96.1 (77.6)    |
| Redundancy     | 4.5 (2.5)       | 4.1 (2.2)                   | 5.0 (3.0)                          | 4.3 (3.1)                   | 2.7 (1.6)      |

### Figures

**Figure 1** High-resolution APE1–DNA product complex. (a) Overview of APE1–DNA product complex, with APE1 shown in yellow and the 21-mer DNA shown in cartoon representation. The site of cleavage is indicated with a red arrow. The THF, 5′-cytosine (Cy) and orphan base are shown in stick format (gray carbons). (b,c) Focused view of the active site with (b) and without (c) density, shown with key residues and distances (Å) indicated. The protein side chains are shown in yellow and DNA residues in gray. Water molecules and Mg^{2+} are shown as blue and red spheres, respectively. The omit map (green) is contoured at 3σ.
To further probe the role of R181, we conducted kinetic measurements of AP-site incision on the same DNA substrate as in the binding studies. In these experiments, the reaction mixture contained an excess of substrate DNA. The biphasic time courses of product formation demonstrated that catalysis during the first enzymatic turnover was more rapid than in the subsequent steady-state phase for both R181A and wild-type enzymes, thus indicating that a step after chemistry limited enzyme cycling (i.e., product release). For wild-type enzyme, the observed rate constant of the burst phase was 36 s$^{-1}$ and was followed by an apparent linear rate ($v_{ss}$) of 9 nM/s. With this analysis, the burst amplitude represented the apparent active enzyme concentration (11 nM) so that the steady-state rate ($k_{ss} = v_{ss}/[\text{APE1}_{\text{active}}]$) was 0.8 s$^{-1}$. For the mutant enzyme, the observed rate constant of the burst phase was 14.2 s$^{-1}$ and was followed by an apparent linear rate ($v_{ss}$) of 20 nM/s corresponding to a steady-state rate of 1.9 s$^{-1}$. Because the observed burst and steady-state rates for the mutant enzyme were not well separated (i.e., $k_{\text{burst}}/k_{ss} < 10$), we used them to calculate $k_{\text{incision}}$ and $k_{\text{dissociation}}$ for the mutant enzyme, which were 11.9 and 2.3 s$^{-1}$, respectively (Online Methods). This represents a three-fold decrease in the incision rate and a three-fold increase in the product dissociation rate constant for the mutant enzyme (Fig. 2c and Supplementary Table 1). Together, these binding and kinetic results indicate that R181 facilitates product DNA binding.

An essential aspect of the reaction mechanism is the flipping of the AP site into the active site, leaving an orphan base in the opposing strand (Fig. 1a). R177 acts as a surrogate base by intercalating into the major groove, thus forming a base-stacking interaction (Fig. 3a). Previously reported structures have shown R177 contacting only the backbone nonbridging oxygen of the AP site in both the product and substrate complexes\textsuperscript{11}. Those putative interactions do not adequately account for the differential loss in substrate and product binding reported for the R177A mutant (7- and 500-fold decrease, respectively)\textsuperscript{27,28}. Instead, product-specific stabilization by R177 can be rationalized from our product structure. This structure indicates that R177 comes within hydrogen-bonding distance of the orphan base and a water molecule that can form a hydrogen bond to the backbone of the cleaved AP site (Fig. 3b). These contacts are specific to product DNA, according to an overlay of the substrate and product complexes. The overlay indicates that R177 swings 2.0 Å toward the orphan base, and the AP-site backbone moves 1.2 Å away from R177 after cleavage (Fig. 3c). Thus, R177 enhances product binding while only moderately affecting substrate binding.

High-resolution APE1–substrate complexes

The high enzyme concentrations and long incubations necessary for crystallographic studies render enzyme–substrate complexes difficult to trap. The robust incision rate of APE1 and minor reagent Mg$^{2+}$ contamination adds to this difficulty. To overcome these challenges and obtain a substrate complex structure, we used a modified DNA with 2′′-O-methyl phosphorothioate backbone modification 5′ to the AP site. This modification contains a sulfur substitution for a nonbridging oxygen that reduces incision\textsuperscript{6,29}. The resulting substrate complex diffracted to 1.63 Å, a resolution substantially higher than that of a previously reported 3.0 Å substrate complex (Table 1). A difficulty in using the phosphorothioate substrate is the existence of two isomers, $S_p$ and $R_p$. In the crystal structure, we observed both isomers in the active site with equal occupancy (Supplementary Fig. 2). However, the $R_p$ isomer is shifted away from the active site by 2.1 Å (Supplementary Fig. 2b). Only the $S_p$ isomer is in the proper orientation to coordinate key active site residues: N174, Y171, H309 and the nucleophilic water. Therefore, the subsequent description focuses on the structure with the $S_p$ isomer.

The identity of the nucleophile during APE1-mediated cleavage is uncertain. Here we have captured the requisite structural snapshots to identify key catalytic groups involved in APE1 backbone incision (detailed view of the active site with substrate DNA containing the $S_p$ isomer of O-methyl phosphorothioate in Fig. 4). The AP-site backbone oxygen (O5′) is coordinated to N174, whereas Y171 and H309 coordinate the nonbridging oxygen and sulfur, respectively (Fig. 4b). These contacts stabilize the active position of the AP site and are observed in both the product and substrate complexes. The high resolution of our structure allows identification of a water molecule in position to act as the nucleophile (Fig. 4b). This well-ordered water is coordinated by the nonbridging oxygen and sulfur atoms of the backbone phosphate positioning the water 2.8 Å from the

### Table 1

| Substrate       | Wild type ($K_p$ nM) | R181A ($K_p$ nM) |
|-----------------|----------------------|------------------|
|                |                      |                  |
| Product         | 0.4 ± 0.1            | 0.4 ± 0.1        |
|                | 2.7 ± 0.5            | 9 ± 0.6          |
phosphorous atom (Fig. 4c). N212 and D210 oxygen atoms come within hydrogen-bonding distance of the nucleophilic water molecule. In this orientation, D210 (OD1) is poised to activate the water for nucleophilic attack and comes within hydrogen-bonding distance of N68. Additionally, D210 (OD2) is within hydrogen-bonding distance of N212 (backbone nitrogen). This hydrogen-bonding network probably alters the pKₐ of D210, thereby facilitating attack of the nucleophilic water (Fig. 4c).

The substrate complex lacks a clearly identifiable metal ion within the active site even with MgCl₂ present during crystallization. This is consistent with previous structural snapshots indicating high disorder for Mg²⁺ within the active site.²³⁻²⁷ It has previously been shown that APE1 is a metal-dependent enzyme, but the identity of the metal-binding site(s) and the number of metal ions required for catalysis remain controversial.¹³,¹⁸,²⁶ To provide insight into the role of metal binding site(s) and the number of metal ions required for catalysis, we briefly soaked the substrate crystals in a cryosolution containing MnCl₂ in an equimolar concentration to MgCl₂ (Table 1) and determined the 1.8-Å substrate complex with both the nucleophilic water and Mn²⁺ ion bound within the active site (Fig. 4d). We verified the presence of Mn²⁺ by its anomalous signal (Supplementary Fig. 3a). This Mn²⁺ coordinates D308, E96, D70 and three water molecules (Fig. 4d). One of these coordinating water molecules is within hydrogen-bonding distance to the nonbridging sulfur. This positions Mn²⁺ only 2.7 Å from the location of the metal in the product structure (Supplementary Fig. 3c). Overlaying the metal-bound and metal-free substrate complexes indicated that E96 and D70 shift 1.2 and 1.9 Å, respectively, upon metal binding, (Supplementary Fig. 3b). The nucleophilic water remains in the same position in both substrate complexes and is located 7 Å from Mn²⁺. The position of this metal-binding pocket near the site of cleavage probably facilitates catalysis and stabilizes reaction intermediates after bond breakage.

APE1 processing an AP site with a 5′ mismatch

The presence of a mismatch 5′ to the AP-site lesion has been shown to dramatically reduce the catalytic activity of APE1 (refs. 29,30). A biologically relevant mismatch that APE1 may encounter during BER is a T–G mismatch arising from deamination of the epigenetic marker 5-methylcytosine in CpG dinucleotide repeats in which the guanine

**Figure 3** R177 intercalates into the major groove anchoring the orphan base. (a) Arginine stabilization of the product complex. The DNA in our product complex excluding the THF is shown in a transparent gray surface representation. R177 and M270 are shown in yellow stick format, intercalating into the major and minor groove, respectively. A structural water is shown as a blue sphere within this cavity. (b) The same orientation as in a, with the orphan base and THF shown in yellow. An omit map contoured at 3σ is shown for R177, and the distances (Å) are indicated. (c) Overlay of the previous substrate (PDB 1DE8 (ref. 11)) and our product complex, shown with key residues in blue or yellow, respectively. The DNA in gray corresponds to our product complex. Shifts after product formation are shown with red arrows, with the distances (Å) indicated.

**Figure 4** High resolution APE1–DNA substrate complex. (a,b) The APE1 active site with (a) and without (b) density, with key residues, cleavage point and distances (Å) indicated. The protein side chains are shown in salmon and DNA in gray. The nucleophilic water is shown as a blue sphere. The omit map (green) is contoured at 3σ. (c) Focused view of the nucleophilic water, with the inline attack shown with a red arrow. The distance (Å) between the nucleophilic water and phosphate group is indicated. Key contacts promoting the activation of the water molecule are highlighted with dashes. (d) The complete APE1 active site after soaking in MnCl₂, with key residues and distances indicated. The protein side chains are shown in green and DNA in gray. The Mn²⁺ and water ions are shown in purple and blue, respectively.
Figure 5 APE1 E96Q D210N mismatch substrate complex. (a) Close-up of the mutant APE1 active site, with key amino acid residues indicated. The 5′-thymidine (Ty) (relative to THF) mispaired with guanine adopts a wobble conformer (cyan) and Watson-Crick (WC)-like mismatch conformer (gray), as indicated. The backbone shift in the wobble conformer is indicated by dashes. (b) Focused view of the wobble and WC-like conformer, shown in cyan and gray, respectively. The omit map is contoured at 3σ for the T-G mispair. The phosphate backbone location of each conformer is indicated. (c) Top, T-G wobble base-pairing interaction. Bottom, WC-like mismatch conformer. The distances (Å) between base-pairing groups are indicated. (d) Same view as in a, with the inactive wobble base pair removed. The distances (Å) to key active site residues and the nucleophilic water are shown.

is damaged (for example, 8-oxoguanine) and undergoing repair. Previous kinetic studies have shown that a 5′ T-G mismatch reduces AP-site incision by ~5,400-fold. Currently, the molecular basis for this reduction remains unclear. To probe this catalytic defect, we characterized a catalytically dead double-mutant enzyme (E96Q D210N) in combination with a natural DNA substrate containing a T-G mismatch 5′ to the AP-site lesion. The resulting crystal diffracted to 1.8 Å and showed APE1 bound to substrate DNA in a similar global conformation as that observed with correctly base-paired DNA and wild-type APE1 (Table 1). Overlaying the wild-type substrate complex with the double mutant provided insight into how this enzyme binds DNA with similar affinity but is unable to incise an AP site. The Q96, N68 and N210 residues form a catalytically dead triad by undergoing moderate rotameric shifts to hydrogen-bond with one another (Supplementary Fig. 4). This prevents E96Q from coordinating the metal, because NE2 is pointed toward the metal-binding pocket, and OE1 coordinates ND2 of N68. A similar phenomenon occurs with the N210 substitution. N210 (ND2) coordinates OD1 of N68, thus resulting in ND2 coordinating the nucleophilic water molecule. The nucleophilic water molecule is in the same location as observed with the phosphorothioate substrate complex but is unable to be activated by N210 (Supplementary Fig. 4b). This combination prevents APE1 from cleaving DNA, thus validating that the substrate contacts observed in the phosphorothioate complex are not artifacts of the modified DNA.

A close-up view of the active site shows the T-G mispair being accommodated in the substrate complex by forming either a wobble or Watson-Crick (WC)-like mismatch within a single structural snapshot (Fig. 5a–c). This conformer is probably not active, because the backbone phosphate is shifted 1.8 Å out of the active site (Fig. 5a). In contrast, the WC-like mismatch is formed through either an ionic or tautomeric interaction between the bases (Fig. 5c). The WC-like mismatch represents the active conformer because the phosphate backbone remains within the active site (Fig. 5a). In this active conformer, the nucleophilic water is 2.5 Å from the phosphate and only 2.7 and 2.5 Å from its nonbridging oxygens (Fig. 5d). In addition, key active site contacts with Y171 and N174 are not within a stable hydrogen-bonding distance of the phosphate backbone (Fig. 5d). The loss of these contacts and alternate conformers of the T-G mispair reduce the cleavage activity of APE1.

To capture the product state, we used wild-type APE1 to form the product complex containing a 5′-T-G mispair (Table 1). We observed density corresponding only to the product complex after cleavage (Fig. 6a). The key contacts to the product phosphate with

Figure 6 APE1 T-G mismatch product complex. (a, b) The APE1 active site after cleavage with a T-G mismatch 5′ to the THF, shown with (a) and without (b) density. Water and magnesium ions are shown as blue and red spheres, with key residues, site of cleavage and distances (Å) indicated. (c) Omit map density (3σ) for the wobble T-G base pairing, shown with distances (Å). (d) Overlay of the APE1 product complex with a 5′ matched and mismatched base pair in yellow and magenta, respectively. The base-pairing interaction for the T-G wobble base pair is shown as dashes, and the shift arising from the mismatch is highlighted with a red arrow with the corresponding distance (Å). The Mg²⁺ and key residues for each structure are indicated.
N174, Y171, D210, N212 and H309 are maintained in the product complex (Fig. 6b). The phosphate backbone also coordinates a single Mg\(^{2+}\). E96 and three water molecules that are coordinated by D70 and D308 coordinate this Mg\(^{2+}\). In this complex, in contrast to the substrate complex, the T-G mispair adopts only a wobble base upon cleavage of the phosphate backbone (Fig. 6c). This wobble base-pair conformation results in a 3.0-Å shift of O3′ compared to the product complex without a mismatch (Fig. 6d), thereby removing O3′ from the active site Mg\(^{2+}\) coordination sphere in the product-mismatch complex (Fig. 6b). The shift to wobble base-pairing indicates that the strain within the active site is not relieved until backbone incision.

**DISCUSSION**

APE1 has been implicated in a number of critical biological pathways such as BER, RNA cleavage, 3′-end processing, redox regulation, cell proliferation and nucleotide incision repair\(^{37-42}\). In agreement with a critical biological role for this enzyme, mouse model studies have indicated that APE1 gene deletion is embryonic lethal\(^{43}\). Even subtle APE1 point mutations or alterations in expression levels have been associated with the development of cancer, aging and cardiovascular and neurological diseases\(^{44,45}\). Some aggressive cancers show increased expression of APE1, which is presumed to protect metabolically active cancer cells from the high level of reactive oxygen species during enhanced oxidative phosphorylation\(^{46}\). This protective effect has resulted in APE1’s emergence as a therapeutic drug target to reduce enzymatic activity during chemotherapy\(^{41}\).

The lack of APE1 active site structural detail has resulted in mechanistic ambiguity\(^{11,14,15}\). The prechemistry structures reported here indicate that a water molecule is in position for an inline nucleophilic attack in all three substrate complexes, even with mutant APE1 or modified DNA. This location is similar to that of an ordered water molecule previously reported in an apoenzyme APE1 structure\(^{26}\); modified DNA. This location is similar to that of an ordered water molecule that contacts the nonbridging oxygen of the phosphate. Indeed, the nucleophilic water is positioned for an inline attack on the phosphate backbone by hydrogen-bonding to a water molecule.

![Image](46x622 to 213x720)

**Figure 7** APE1 mechanism during strand cleavage. (a) Organization of the active site before the attack by the nucleophilic water (H\(_2\)O\(_{nuc}\)), shown with a red arrow indicating the orientation of attack. The cleavage point, key side chains and AP site are indicated. The Mn\(^{2+}\) (purple) and water (blue) molecules are shown as spheres. The location of the free electron pairs for the nucleophilic water are shown as a yellow bar with black dots for the electrons. These locations represent the distributed cloud determined by Guassian\(^{57}\). Locations of the proton atoms are shown as light blue spheres with an H on the dashed lines. (b) Proposed refined APE1 mechanism based on our high-resolution structural snapshots. Key observations are highlighted in blue. The ground and product states are based on our structural snapshots. The transition-state model is inferred from the two structures.
nonbridging oxygen and the leaving-group oxygen that becomes O3’. During or immediately after cleavage, the proton from the nucleophilic water molecule is transferred to D210 and is subsequently released to solvent. The final product state results in a 5’-sugar phosphate and 3’-hydroxyl that are coordinated by Mg²⁺, N174, D70, E96, H309, Y171 and N212. N212 rotates, thereby providing a hydrogen-bond donor and stabilizing the product state. The interactions observed here appear to be fundamental to catalysis, because alterations that eliminate each of these interactions strongly diminish catalytic activity.

The epigenetic control of gene expression occurs in tracts of CpG dinucleotides through the methylation of cytosine to form 5-methylcytosine51. In mammalian promoters containing CpG tracts, 70–80% of the cytosines undergo methylation52. These modified 5-methylcytosine residues will often undergo spontaneous deamination, thereby precisely positioning the phosphate backbone into the active site. This is consistent with the rigid platform that APE1 provides upon binding the DNA and the need to precisely position the DNA into the active site; this mechanism optimizes APE1 for correctly base-paired DNA flankng the AP site, and DNA abnormalities reduce the rate of incision. Because APE1 must deal with AP sites in a large number of DNA contexts, it may have evolved a rapid incision rate to provide a kinetic buffer to overcome situations in which it must incise an AP site in a less than ideal situation.

Given the high number of AP sites in the genome, it is essential that they be rapidly processed to maintain genomic integrity. The rapid APE1 incision rate is facilitated by use of water as a nucleophile and a preformed metal-binding site to stabilize the developing charge during the course of the reaction. However, incision generates a cytotoxic DNA break that could lead to chromosomal rearrangements. To prevent this, APE1 has evolved a slow product-release step that enables channeling to other DNA-repair enzymes56.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 5DFF (product complex), 5DF1 (phosphorothioate substrate complex), 5DFJ (E96Q D210N mismatch substrate complex), 5DFH (mismatch product complex) and 5DG0 (phosphorothioate substrate complex with Mn²⁺).

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank the Collaborative Crystallography group at NIEHS for help with data collection and analysis. We thank L. Pedersen and L. Perera for valuable discussions. This research was supported in part by the Intramural Research Program of the US National Institutes of Health, National Institute of Environmental Health Sciences (project numbers Z01-ES050158 and Z01-ES050161 (S.H.W.)). A part of this research was performed at Oak Ridge National Laboratory’s Spallation Neutron Source and the Joint Institute for Neutron Sciences Biophysical Characterization Laboratory, sponsored by the United States Department of Energy, Office of Basic Energy Sciences (M.J.C.). N.S.D. is supported in part by Eli Lilly and Co. and the United States Department of State, as part of the United States-Russia collaboration in the Biomedical Sciences US National Institutes of Health Visiting Fellows Program.

**AUTHOR CONTRIBUTIONS**

B.D.F. designed the project. B.D.F. carried out crystallography. N.S.D. did the kinetic analyses. M.J.C. did the binding studies. B.D.F., W.A.B. and S.H.W. prepared the manuscript. All authors discussed the results and commented on the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS
DNA sequences. To generate the 21-mer for crystallization, the following DNA sequences were used (IDT): nondenatured strand, 5′-GGA-TCC-GTC-GGG-GGC-ATC-ACC-3′; modified matched strand, 5′-GCT-GAT-GGC-CGC-GAC-GGA-TCC-3′, where the underlined X represents THF; modified mismatched strand, 5′-GCA-GTC-GCC-GAC-CGG-GAT-GCC-3′, with a 2′O-methyl phosphorothioate-containing strand, 5′-GC-TCT-GCC-CTT-TGG-CCA-GAC-GTT-3′. The substrate was formed with a 46-mer, 5′-GGA-TCC-3′′. The substrate was generated by allowing APE1 to cleave the DNA (MgCl2 in annealing buffer) with molecular replacement with two APE1 molecules (PDB 1DEW11), and the DNA was built with Coot. Refinement was carried out with PHENIX and model building with Coot. The metal-ligand coordination restraints were generated by ReadySet (PHENIX) and not used until the final rounds of refinement. The figures were prepared in PyMOL (http://www.pymol.org/), and all density maps were generated after performing simulated annealing. Ramachandran analysis determined that 100% of non-glycine residues were in allowed regions, and at least 96% were in favored regions.

Kinetic characterization. A rapid quench-flow system was used for activity measurements. The fluorescein-containing dumbbell DNA substrate containing THF (F) was used to measure APE1 incision activity (37 °C). The reaction buffer was 50 mM HEPES, pH 7.5, 100 mM KCl, 5 mM MgCl2, 0.5 mM EDTA, and 0.1 mg/ml bovine serum albumin. The final concentrations (after mixing) were 100 mM DNA substrate and 30 nM APE1. At time intervals, aliquots were quenched by mixing with 100 mM NaOH. An equal volume of DNA gel loading buffer (10 M urea, 100 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanol) was added to the quenched reaction mixture. After incubation at 95 °C for 5 min, the reaction products were separated by 15% denaturing polyacrylamide gel electrophoresis. A Typhoon phosphorimager was used for gel scanning and imaging, and the data were analyzed with ImageQuant software. The biphasic time courses were fit to the equation: product = A(1 − e−kobs,t) + v∗t, where A represents the amplitude of the rising exponential and kobs is the first-order rate constant. The steady-state rate constant (kcat) is the steady-state velocity (v∗)/A, where A represents the fraction of actively bound enzyme. When the observed burst and steady-state rate constants are not well separated (i.e., kcat/koff < 10), these values do not represent intrinsic rate constants. The intrinsic rate constants can be calculated from kobs = (kcatincision + koffproduct) and kcat = (kcatincision × koffproduct)/(kcatincision + koffproduct).

Anisotropy binding studies. Fluorescence anisotropy measurements were used to quantify binding of wild-type APE1 and the R181A mutant to a DNA dumbbell as described above. Fluorescence anisotropy measurements were carried out on a Horiba Fluorolog Fluorimeter at 20 °C in a buffer consisting of 50 mM Tris-HCl, pH 7.0, 25 mM NaCl, 10 mM EDTA and 0.1 mM DTT. The excitation and emission wavelengths were 485 and 520 nm, respectively, each with a 14-nm slit width. For all DNA titrations, concentrations of the fluorescein-labeled DNA dumbbell ranged from 0.5 mM to 10 nM and were adjusted depending on the initial Kd value determinations to be below the Kd. The lower limit (0.5 mM) was based on instrument configuration limitations, and insufficient signal to noise was observed when the DNA probe was below this level. Fluorescence anisotropy changes were normalized to the number of binding sites and fit to either a one-site binding model or to a two-site binding model with Origin (ref. 62). A minimum of three independent titrations was averaged to determine Kd values.

Protein expression and purification. Human wild-type APE1 was expressed from the pxc53 clone described previously59. The truncated APE1 clone was generated from pxc53 through removal of the N-terminal 43 amino acids by PCR. All mutagenesis was carried out in either the full-length or truncated clones. APE1 was expressed in E. coli BL21(DE3) and induced as previously described59. After induction, the cells were lysed by sonication in ice. The enzyme was purified with a HisTrap column with a linear gradient of NaCl up to 1 M. APE1 eluting at 4 °C was collected and concentrated. The concentration was determined by absorbance at 260 nm.

Crystallography. Human wild-type APE1 was expressed in the rising exponential and kobs to product)/[(kcatincision × koffproduct)/(kcatincision + koffproduct)].