PRE-STeady-state KINETIC CHARACTERIZATION OF THE AP ENDONUCLEASE ACTIVITY OF HUMAN AP ENDONUCLEASE 1
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Running title: Pre-steady-state AP endonuclease activity of APE1

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Human AP endonuclease 1 (APE1, REF1) functions within the base excision repair pathway by catalyzing the hydrolysis of the phosphodiester bond 5’ to a baseless sugar (apurinic or apyrimidinic site). The AP endonuclease activity of this enzyme and two active site mutants were characterized using equilibrium binding and pre-steady-state kinetic techniques. Wild-type APE1 is a remarkably potent endonuclease and highly efficient enzyme. Incision 5’ to AP sites is so fast that a maximal single-turnover rate could not be measured using rapid mixing/quench techniques and is at least 850 s⁻¹. The entire catalytic cycle is limited by a slow step that follows chemistry and generates a steady-state incision rate of about 2 s⁻¹. Site-directed mutation of His-309 to Asn and Asp-210 to Ala reduced the single turnover rate of incision 5’ to AP sites by at least 5 orders of magnitude such that chemistry (or a step following DNA binding and preceding chemistry) and not a step following chemistry became rate-limiting. Our results suggest that the efficiency with which APE1 can process an AP site in vivo is limited by the rate at which it diffuses to the site, and that a slow step after chemistry may prevent APE1 from leaving the site of damage before the next enzyme arrives to continue the repair process.

Human apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional protein that is required for cell viability. APE1 is an endonuclease that functions within the base excision repair (BER) pathway to initiate the repair of abasic (AP) sites in DNA (reviewed in (1,2)). The BER pathway is a series of enzymatically catalyzed reactions used by cells to remove and replace damaged or lost bases (reviewed in (3)). APE1 catalyzes the hydrolysis of the sugar-phosphate backbone of DNA 5’ to an AP site. The 3’ end of the nick generated in the damaged DNA strand can then be extended by a DNA polymerase to replace the damaged or missing base.

Human APE1 is homologous to E. coli EXOIII (4,5) and like EXOIII possesses 3’-5’ exonuclease activity as well as 3’ phosphatase, and 3’ phosphodiesterase activities (6). Unlike E. coli EXOIII, the AP endonuclease activity of human APE1 is far more robust than any of its other repair activities (7). APE1 also has several other functions in the cell beyond its DNA repair functions and has been shown to stimulate the DNA binding activity of certain transcription factors (AP-1 (FOS /JUN), Egr-1, NF-kb and p53). APE1 can act as a transcription factor itself and bind to promoters containing negative Ca²⁺ response elements (nCaRE sites) (8,9). Finally, a truncated form of APE1 with increased 3’ to 5’ exonuclease activity and increased endonuclease activity on undamaged DNA has been implicated in the fragmentation of the genome associated with apoptosis (10,11).

All of the above functions of APE1 have been found to be essential for cell viability and/or cell division and differentiation (12,13). APE1 null mice or mouse ES cells could not be made because these embryos had significant developmental flaws and lost viability at about embryonic day 5.5 (14). Studies using RNA interference to reduce the levels of APE1 in several human cell types have shown that reduction of APE1 expression halted cell proliferation, resulted in an accumulation of AP sites in DNA, and stimulated cell death via apoptosis (15).

Although the role of APE1 in base excision repair has been fairly well defined, the mechanism by which APE1 catalyzes the hydrolysis of the phosphodiester bond 5’ to an AP site has not been characterized as extensively. Questions as simple as how fast the hydrolysis reaction is remain to be
answered. Presumably, hydrolysis of the phosphodiester bond requires mechanisms for 1) activating a water molecule or perhaps another nucleophile for attack on the phosphorayl group, 2) stabilizing charge that builds up on what is most likely a pentameric phosphoryl transition state, and 3) donating a proton to the leaving group (3’ alkoxide). Divalent cations could perform some of these functions and are required for the AP endonuclease activity of APE1, but the number required and their role in catalysis is not clear. Depending on the pH, crystalline forms of the enzyme contain either 1 or 2 divalent cations in the active site (16-18). Amino acid residues serving as general acids and/or general bases could also perform these functions in catalysis, and structural and mutagenesis studies have suggested that His-309 and Asp-210 could function in this capacity. His-309 has been proposed to hydrogen bond to an oxygen on the phosphoryl moiety to stabilize charge in the transition state and mutation of His-309 to Asn reduces steady-state AP endonuclease rates by 30,000-fold (19,20). Two roles for Asp-210 have been proposed. First, it may activate a water molecule for attack on the phosphoryl, and second it may subsequently protonate the 3’ alkoxide leaving group. Mutation of Asp-210 to Ala reduces the steady-state AP endonuclease activity by 25,000-fold (21,22). As an initial step in defining the kinetic mechanism of APE1 and the contributions of two amino acid residues, His-309 and Asp-210, to catalysis, pre-steady-state kinetics of AP site incision and equilibrium binding to AP-site-containing DNA were measured for wild-type and mutant enzymes.

**EXPERIMENTAL PROCEDURES**

Preparation of 32P-labeled DNA substrates and APE1 proteins - All substrates were of the sequence 5’GCGTCAAAATGTFGGTATTTCCATG3’ in which F indicates the position of tetrahydrofuran, a reduced abasic site analog. DNA duplexes were labeled on the 5’ end of the damaged strand with 32P and annealed to a complementary oligonucleotide containing a thymine opposite tetrahydrofuran as in (23,24). Mutant and wt APE1 proteins were expressed in and purified from E.coli as described previously (24).

APE1 site-directed mutagenesis - Site-directed mutagenesis was used to generate APE1 mutants with specific amino acid changes in the active site. The pET-14b plasmid containing the APE1 sequence was mutagenized using the QuikChange (Stratagene) site-directed mutagenesis kit, following the manufacturer’s protocol. Histidine 309 was replaced with asparagine (H309N) using two primers of the sequence 5’ccc tcg gca gtg ata act gtc cta ccc t3’ and 5’agg gtg ata gga cag tta ctc cgg agg g3’ (Integrated DNA Technologies). Aspartic acid 210 was replaced with alanine (D210A) using 2 primers of the sequence 5’cct tgt gct gtt tgg acg act cca tgt ggc aca tgg 3’ and 5’cat gtt gta cca cat tga gtt etc cca gca cca ggg 3’ (Integrated DNA Technologies).

Fluorescence anisotropy binding assays - An oligonucleotide 25 nucleotides in length of the sequence 5’GCGTCAAAATGTFGGTATTTCCATG3’ containing tetrahydrofuran, a reduced abasic site analog, indicated by F at position 13 was annealed to a complementary strand that contains a T opposite the AP site. This complement was labeled with X-rhodamine via an amino linker on the 5 position of a thymine 2 nucleotides from the 5’ end. These two strands were mixed in equal molar amounts in 50 mM HEPES, 100 mM KCl, 5% (v/v) glycerol, heated to 80ºC and annealed through slow cooling to room temperature. Anisotropy measurements were obtained using a QuantaMaster QM-1 fluorometer (Photon Technology International) equipped with a 75-watt xenon arc lamp, an excitation monochromator, and dual emission monochromators. The excitation monochromator was set to 580 nm with a band pass of 8 nm leading to the cuvette. The dual monochromators, set in line with the PMTs, were set to 610 nm with a band pass of 8 nm leading to the cuvette. All binding reactions had a final volume of 80 µl. Labeled substrate was diluted into the cuvette in binding buffer (50mM HEPES pH 8, 100 mM KCl, 5% glycerol, 20 mM EDTA) to a final concentration of 20 nM or 40 nM. Anisotropy of the free DNA was calculated according to Equation 1 in which I_v is intensity the vertically polarized emission and I_h is the intensity of the horizontally polarized emission when exciting with vertically polarized light and g (Equation 2) is a correction factor to account for...
differences in the efficiencies of the two detectors. The g factor was calculated from the intensities of vertically, \( I_{vv} \), and horizontally, \( I_{hh} \), polarized emission measured using horizontally polarized excitation light. APE1 was added in various concentrations and the “bound” anisotropy was calculated just as for the free DNA. These data were then globally fit to Equation 3, using Prism (Graphpad software), to determine the \( K_d \) in which \( K_d \) is the dissociation constant, \( E_t \) is the total enzyme concentration, \( D_t \) is the total DNA concentration, \( r_{obs} \) is the observed anisotropy, \( r_b \) is the maximum anisotropy and \( r_f \) is the anisotropy of the unbound DNA.

\[
robs = \frac{I_{vv} - gI_{vh}}{I_{vv} + 2gI_{vh}}
\]

(1)

\[
g = \frac{I_{hv}}{I_{hh}}
\]

(2)

\[
robs = \frac{(K_d + E_t + D_t) - \sqrt{(K_d + E_t + D_t)^2 - 4E_tD_t}}{2Dt}(r_b - r_f) + rf
\]

(3)

**Electrophoretic mobility shift binding assays** - A 25mer oligonucleotide containing tetrahydrofuran was labeled at the 5' end with \(^{32}P\) as described above and annealed to a complementary strand with a thymine opposite tetrahydrofuran. This substrate was then mixed at room temperature with various amounts of APE1 in EMSA buffer (50 mM HEPES pH 8, 100 mM KCl, 10% glycerol, 20 mM EDTA, 0.1 mg/ml BSA). Aliquots of the binding mixtures were loaded into a 6% non-denaturing polyacrylamide gel. Polyacrylamide electrophoresis was performed at 4 °C at 8 V/cm² for 90 min. Gels were visualized and quantified using the Storm phosphorimager and ImageQuant analysis software (Amersham Biosciences). Data were fit to Equation 4 using SigmaPlot (Systat Software) to determine the \( K_{dapp} \) in which \([ED]\) is the concentration of the DNA bound by the enzyme, \( K_{dapp} \) is the apparent dissociation constant, \( E_t \) is the total enzyme concentration and \( D_t \) is the total DNA concentration.

\[
[ED] = \frac{(K_{dapp} + E_t + D_t) - \sqrt{(K_{dapp} + E_t + D_t)^2 - 4E_tD_t}}{2}
\]

(4)

**APE1 AP endonuclease activity assays** - APE1 endonuclease activity assays were performed using the KinTek RQF-3 rapid quench instrument (KinTek) in constant quench mode. APE1 and tetrahydrofuran containing DNA were mixed in a reaction buffer containing 50 mM HEPES pH 8, 100 mM KCl, 5% (v/v) glycerol, 5 mM MgCl₂ and 0.2 mg/ml BSA. Reactions were quenched using 0.2 M NaOH. Two volumes of 96% formamide / 20 mM EDTA were added to quenched reactions, which were then heated to 95°C for 10 minutes. Substrates were separated from products by denaturing PAGE on 12% acrylamide/8M urea gels. Gels were visualized and quantified using a Storm phosphorimager and ImageQuant analysis software (Amersham Biosciences). The concentration of sodium hydroxide necessary to effectively quench the reaction was determined empirically by titrating the quencher. In one series of experiments done in duplicate, reactions containing 20 nM TXG and 1000 nM APE1 were quenched after 2 ms with 0, 0.05, 0.1, 0.2, 0.3, and 0.4 M NaOH. The percent substrate converted to product was 98.8% whereas the sodium hydroxide quencher stopped reactions with about the same efficiency at all concentrations tested. Concentrations of 0.05 and 0.1 M NaOH limited the percent substrate converted to product to 80.8 ± 0.2%, and concentrations of 0.2, 0.3, and 0.4 M NaOH limited product formation to 79.3 ± 0.5%. AP endonuclease activity assays for the APE1 mutants were done using hand-mixing techniques with the same buffers and conditions used in the assays for the wild-type enzyme.

Multiple-turnover assays for wild-type APE1 were fit by a single exponentials plus linear steady-state phases (Equation 5). Single-turnover data for wild-type APE1 were fit to double exponentials (Equation 6) and single-turnover data for APE1 mutants were fit to single exponentials (Equation 7). Curve fitting was done using SigmaPlot (Systat Software) and figures were made using Kaleidagraph (Synergy Software).

\[
y = a(1 - e^{-kt}) + st
\]

(5)
\[
y = a(1 - e^{-k_1t}) + b(1 - e^{-k_2t}) \\
y = a(1 - e^{-kt})
\]

RESULTS

Equilibrium binding to AP-site DNA - A fluorescence-anisotropy-based binding assay was employed to determine the binding affinity of APE1 for DNA duplexes containing an AP site (AP-DNA). Duplex DNA contained a single reduced abasic site analog, tetrahydrofuran, at position 13. APE1 binds both natural and reduced AP sites and catalyzes hydrolysis of the 5'-phosphodiester bond (25). The reduced AP site was used in these assays because it is less reactive and more stable than the natural AP site. Duplexes were covalently labeled with X-rhodamine (RhX) via an amino linker on the 5'-position of a thymine located on the strand complementary to the AP-containing strand, 2 nucleotides from the 5'-end. Binding of wt APE1 to AP-DNA was determined by measuring the anisotropy of the RhX probe in assays containing either 20 or 40 nM AP-DNA in the presence of 20 mM EDTA to inhibit strand incision. Given that the APE1-AP-DNA complex is larger and tumbles more slowly than free AP-DNA, the anisotropy of the RhX reporter increases as the fraction of bound DNA increases (26). Binding data from four independent experiments were globally fit to a single dissociation constant (Equation 3) yielding a \(K_d\) value of 11 ± 2 nM for wt APE1 (Fig. 1). Binding to undamaged DNA was not detectable under conditions used in Fig. 1 (data not shown), and therefore this \(K_d\) value reflects a specific interaction with AP-DNA.

Multiple-turnover AP-site incision assays – Incision 5' to a reduced AP site by APE1 was measured under pre-steady-state multiple-turnover conditions in which AP-DNA was present in excess over enzyme. AP endonuclease reactions were initiated by rapid addition of APE1 to \(^{32}\)P-labeled DNA as described in the Experimental Procedures. Time courses for product formation were measured by rapidly quenching reactions in sodium hydroxide after different incubation times, separating DNA substrates from products by denaturing PAGE, and phosphorimaging. Two series of reactions were done. One series (Fig. 2, upper panel) contained 40 nM APE1 and 80, 160, 250, 350, or 500 nM AP-DNA, and the second series (Fig. 2, lower panel) contained 200 nM APE1 and 500, 1000, or 2000 nM AP-DNA. Time courses for both series of reactions gave a rapid burst of product followed by a linear increase in the amount of product. This kinetic behavior shows that the first round of conversion of substrate to product occurs rapidly, but that some step following the chemical step limits the rate at which APE1 can dissociate from product and catalyze subsequent rounds of incision. The overall rate of product formation in the rapid pre-steady-state phase of the reaction increased with increasing DNA concentrations. However, due to difficulty in quantitating the relatively small fraction of substrate converted to product in the burst phase of the reactions, particularly at high DNA concentrations, rates obtained from empirical fits of these data (solid curves shown in Fig. 2) to an exponential plus a linear rise (Equation 5) contain considerable error. That being said, these fits indicated that the pre-steady-state burst rate was greater than 200 s\(^{-1}\) in reactions containing 200 nM APE1 and 500 nM AP-DNA (Fig. 2 legend). Steady-state rate constants for the linear phase of product formation were 1.4 ± 0.1 and 2.3 ± 0.4 s\(^{-1}\) for experiments done at 40 and 200 nM APE1, respectively, or about 2 orders of magnitude slower than the observed rate for the pre-steady-state burst.

Another notable feature of both series of reactions was that the burst amplitudes were less than one enzyme equivalent of product. The amplitudes for reactions containing 40 nM APE1 are just under 30 nM, and the amplitudes for reactions containing 200 nM APE1 are less than 150 nM. One explanation for burst amplitudes that are less than one enzyme equivalent in multiple-turnover reactions is that the total protein concentration determined from the absorbance at 280 nm (see Experimental Procedures) is greater than the concentration of active enzyme.

APE1-AP-DNA binding under stoichiometric conditions - To test possibility that the active site concentration of APE1 was less than the total protein concentration, binding of APE1 to AP-DNA was measured under stoichiometric conditions using the RhX-anisotropy-based assay. Binding reactions contained 1000 nM AP-DNA in the presence of 20 mM EDTA to inhibit the incision reaction. Fig. 3 shows the average results...
for two independent experiments. The increase in anisotropy due to APE1-AP-DNA binding reaches a maximum at a concentration of about 1000 nM APE1 or an enzyme:DNA ratio of about 1:1 and does not increase further. The simplest interpretation of this result is that the enzyme is at least 90% active and the enzyme binds AP-DNA as a monomer. This result suggests that the reduced burst amplitudes in multiple-turnover incision reactions may not stem from a reduction in the active site concentration relative to the total protein concentration.

**Single-turnover AP-site incision assays** – Another explanation for burst amplitudes less than one enzyme equivalent in multiple-turnover experiments is an internal equilibrium between enzyme-substrate and enzyme-product complexes that arises from a significant rate of the reverse reaction relative to the forward reaction (27). To test this possibility and to determine the maximal rate of incision 5’ to AP sites, the AP endonuclease reaction was measured under single-turnover conditions with enzyme in excess of DNA. Reactions contained 0.02 μM AP-DNA and APE1 concentrations ranging from 0.04 μM to 6 μM (Fig. 4), and were initiated by rapid addition of AP-DNA to APE1. The overall rates of the AP endonuclease reactions increased with increasing enzyme concentrations, however, analysis of individual reaction progress curves showed that each was composed of two phases and was best fit by a double exponential rise to a maximum (Equation 6 and solid curves through data points in Fig. 4). Data from a second independent series of single-turnover experiments gave the same results and are presented separately in Supp. Fig. 1 for clarity. The rate of the rapid phase increased linearly with enzyme concentration until a concentration of about 2 μM APE1 at which point rates became too fast to measure experimentally (Figs. 4 and 5). Given that the observed rates are a function the rates of both enzyme-DNA binding and formation of product in the enzyme active site (Fig. 5, steps a and b in the reaction scheme), a linear increase in rate with enzyme concentration indicates that enzyme-DNA binding and not chemistry is rate-limiting over this concentration range. A fit of the observed rates of the rapid phase as a function of enzyme concentration to a line (Fig. 5) yielded an apparent on-rate constant (slope) of 3.5 x 10^8 M^-1s^-1 and an apparent off-rate constant (y-intercept) 47 s^-1 (27). At concentrations greater than 2 μM APE1, about 70% of the substrate was consumed in the shortest time, 2 ms, that could be measured and the majority of the rapid phase was already complete. This rapid reaction precluded determination of a maximal single-turnover rate, but it must be at least 850 s^-1 to give rise to these data.

The amplitudes of each kinetic phase were also a function of enzyme concentration. At the lowest APE1 concentration, 0.04 μM, the amplitudes for the rapid and slow phases were about the same. As the APE1 concentration increased, the fraction of product turned over in the rapid phase, as measured by the amplitude, increased, whereas the amplitude of the slow phase decreased (Fig. 4 and Supp. Fig. 1). At APE1 concentrations greater than 2 μM, the amplitudes no longer changed with increasing APE1 concentrations and reached maximum values of about 16 nM product formed in the rapid phase and 4 nM in the slow phase for reactions that contained 20 nM AP-DNA substrate. The biphasic nature of the single-turnover kinetic data is consistent with an internal equilibrium being established between enzyme-substrate and enzyme-product that requires an “irreversible” step to drive the reaction towards completion. The fraction of product formed in the rapid phase reflects product produced in the enzyme active site (EP), and the slow phase reflects the step that drives the pathway forward by limiting the reverse reaction. The observed rates of the slow phase showed a smaller dependence on enzyme concentration, and increased from a value of about 4 s^-1 at 0.4 μM APE1 to a maximum value of about 20 – 30 s^-1 at APE1 concentrations of 0.5 μM and greater.

**Effects of mutation of His-309 to Asn on APE1 DNA binding and AP endonuclease activity** - Binding of APE1-H309N to the same AP-DNA substrate used for wt APE1 in Fig. 1 was measured using the anisotropy-based binding assay. A K_d value of 53 +/- 7 nM was calculated from data shown in Fig. 6A, a value about 5-fold greater than that for wt APE1.

Single-turnover AP-site incision assays were performed for APE1-H309N as described for those with wt APE1 (Fig. 4), however, reactions catalyzed by the mutant were so slow that assays could be done by “hand-mixing” rather than using
a rapid-mixing apparatus. Results from experiments done using 20 nM AP-DNA and 100 – 1000 nM APE1-H309N are shown in Fig. 6B. In contrast to reactions with wt APE1, the rate of incision 5’ to the AP-site reached a maximal value at saturating APE1-H309N concentrations. A fit of these data to a single exponential (Eq. 7 and Fig. 6B, solid lines through data) yielded a maximal rate of 0.001 s⁻¹.

**Effects of mutation of Asp-210 to Ala on APE1 DNA binding and AP endonuclease activity** – Binding of APE1-D210A to AP-DNA was measured in the anisotropy-based equilibrium binding assay. A $K_d$ value of 2.4 +/- 1.3 nM was calculated from the binding isotherm shown in Fig. 7A. Given that the $K_d$ value was about 10-fold lower than the absolute DNA concentration used in the binding assay, it was desirable to repeat the measurement at a lower DNA concentration to confirm this value. Because the fluorescence at low DNA concentrations was relatively weak, these experiments were done using 32P-labeled AP-DNA in an electrophoretic mobility shift assay. Radio-labeled AP-DNA (5 nM) was incubated with APE1-D210A at concentrations ranging from 1 – 20 nM in the presence of EDTA. Bound DNA was separated from free DNA by electrophoresis on nondenaturing polyacrylamide gels and quantitated by phosphorimaging. A fit of these data (Fig. 7B) to a quadratic equation (Eq. 4) yielded a $K_d$ value of 1.5 +/- 0.4 nM in good agreement with the value of 2.4 +/- 1.3 nM obtained from the anisotropy-based assay. These results show that APE1-D210A binds AP-DNA with about 5-fold greater affinity than wt APE1 and about 25-fold greater than APE1-H309N.

APE1 DNA binding and AP endonuclease activity was measured in single-turnover assays as done for APE1-H309N in reactions containing 20 nM AP-DNA and up to 20 µM APE1-D210A. In the case of the D210A mutant, the incision reaction was much slower and less than half the substrate was converted to product in 23 hours (*data not shown*). Rates were not calculated based on these experiments because of the prolonged incubation times required and the possibility that loss of enzyme activity during the extended reaction could contribute to the kinetics.

**DISCUSSION**

APE1 is the major enzyme in human cells responsible for initiating the repair of AP sites in DNA. Although much work has been done in characterizing the AP endonuclease activity under steady-state conditions, this is the first study to investigate the pre-steady-state kinetics of incision 5’ to AP sites by APE1. This work showed that the first turnover of substrate by APE1 is rapid and limited by the rate of diffusion of the enzyme to DNA. The steady-state turnover is significantly slower, at least 2 orders of magnitude, and is limited by a step after chemistry that controls the rate at which the nicked AP-DNA product is released and the rate at which the enzyme can catalyze another round of incision.

A minimal kinetic model for wild-type APE1 – The simplest possible model to describe this reaction is shown in the scheme in Fig. 5 in which the enzyme binds substrate to form an enzyme-substrate (ES) complex, product is formed in the enzyme active site to produce an enzyme-product (EP) complex, and product dissociates from the enzyme. One biologically relevant question that was addressed by single-turnover kinetic experiments is how fast APE1 can act on an AP site. In these experiments the rate of conversion of ES to EP was so rapid that a maximal rate could not be determined. For this to be the case, the maximal excision rate would have to be at least 850 s⁻¹. The linear dependence of the observed incision rate on APE1 concentration (Fig. 5) demonstrated that the reaction proceeds at a rate that is limited by the rate of enzyme binding DNA and not a subsequent first order reaction such as chemistry. An apparent on-rate of 3.5 x $10^8$ M⁻¹ s⁻¹, calculated from these data, is consistent with a diffusion limited rate for an enzyme-DNA binding event. Our results are consistent with steady-state studies in other laboratories that yielded $k_{cat}/K_M$ values on the order of $10^7$ - $10^8$ M⁻¹ s⁻¹ which set a lower limit for the binding reaction near the diffusion-controlled limit (21,22). This rapid reaction is amazing given that APE1 binds DNA in a conformation in which the DNA is kinked and the AP site is rotated around the sugar-phosphate backbone and flipped into the enzyme active site (16). Presumably, these conformational changes occur prior to the incision reaction and APE1 either captures the fraction of DNA in
which the AP site is flipped out or binds DNA and facilitates the flipping. Either way, the enzyme is highly efficient for the reaction to occur this rapidly.

Another interesting outcome of these experiments was that the pre-steady-state burst amplitude was less than one enzyme equivalent in multiple-turnover assays (Fig. 2). Given that our enzyme preparation is at least 90% active as measured by DNA binding assays (Fig. 3), there are two possible explanations for the reduced burst amplitude. One possibility is that some fraction of the APE1 protein preparation retains DNA binding activity, but has lost (or reduced) AP endonuclease activity. In this case, the concentration of AP endonuclease activity is simply less than the total protein concentration as measured by absorbance at 280 nm or DNA binding. A second possibility is that the reverse of the chemistry step is relatively rapid and an equilibrium between ES and EP is established after the enzyme binds the DNA substrate and before the release of DNA product. This would result in part of the bound substrate being initially converted to product in the burst phase of the reaction. Single-turnover kinetic data provide evidence for the existence of a rapid equilibrium between ES and EP because individual time courses were biphasic, and therefore, we favor the second interpretation. Based on the amplitudes in single-turnover reactions at high APE1 concentrations, about 80% of the bound substrate is converted to product in an EP complex as a result of this internal equilibrium. Complete conversion of substrate to product requires an additional step that depletes EP and that drives the reaction pathway forward such as the product dissociation step in the simple model shown in Fig. 5.

The simple model, however, does not adequately describe all of the AP endonuclease kinetic data. Two lines of evidence suggest that an additional step is present, possibly a step before dissociation of product from EP (Fig. 5, step c). First, modeling single-turnover kinetic data with the simple model in Fig. 5 produces calculated time courses in which the total amount of product at the “endpoint” of the reaction decreases with increasing enzyme concentrations due to the readily reversible reaction pathway. This decrease is not observed in the data and suggests that some step, such as an enzyme conformational change, is likely to be present between formation of product in the enzyme active site (EP) and dissociation of product (E + P) that limits the reverse reaction pathway. Second, the existence of another step is suggested by the observation that the maximal rate of the second phase in single-turnover experiments is about 10 times faster than the steady-state rate in multiple-turnover experiments. If this second phase represented a product release step as in the simple model that drives the reaction towards completion, then the rate of this phase in single-turnover reactions should have approached the steady-state rate in multiple-turnover reactions, but it was faster. This suggests that an additional step is present that gives rise to the faster rate in single-turnover experiments. One possibility is a conformational change that converts the initial [EP] complex to an enzyme product complex, [EP]*, that does not readily undergo the reverse reaction. The relatively small fraction of this slow phase would be apparent in single-turnover reactions in which all the substrate is converted to product in the pre-steady-state phase, but not in multiple-turnover reactions in which a small fraction of substrate is converted product in the first turnover.

APE1 active site mutants – Biochemical experiments with mutants and structural studies have provided evidence that His-309 and Asp-210 are important for catalyzing hydrolysis of the phosphodiester bond 5’ to the AP sites possibly by acting as general acids and/or bases (1,16-18,20-22,28-30). Effects of these mutations were measured on the AP-DNA binding and AP endonuclease activities of APE1. Mutation of His-309 to Asn impaired binding to DNA by about a factor of 5 whereas the APE1-D210A mutant bound AP-DNA about 5 times better than wt APE1. These results are comparable to those based on electrophoretic mobility shift assays which showed that APE1-H309N bound an AP-DNA substrate with about half the affinity as wt (31). Binding measurements were made in the absence of Mg\(^{2+}\) which is required for catalysis, and it is possible that Mg\(^{2+}\) also affects the affinity of APE1 for AP-DNA (29). Both of the residues that were mutated potentially interact with a metal ion in the enzyme active site and this could lead to differences in the relative affinities of the wt enzyme and mutants in the presence of Mg\(^{2+}\) (17).
The effects of the mutations on binding were modest when compared to the effects on the single-turnover kinetics of the AP endonuclease reaction. APE1-H309N required about an hour to process 20 nM AP-DNA whereas wt APE1 could catalyze the same reaction in about 0.2 s, and APE1-D210A was unable to complete the incision reaction in 23 hours. In addition, the rate-determining step in the AP endonuclease reaction changed. For wt APE1, a step following chemistry, possibly product release, was rate-determining, but for the mutants, a step following DNA binding that occurs in the enzyme-DNA complex, possibly the actual hydrolysis reaction, was rate-limiting. A comparison of $k_{cat}$ values underestimates the magnitude of the defect in chemistry due to the mutations because $k_{cat}$ reflects a step after chemistry for wt APE1 and most likely reflects chemistry for the mutants. The $k_{cat}$ value for wt APE1 is about 2000-fold greater than that for APE1-H309N, whereas a comparison of the maximal single turnover rates of at least 850 s$^{-1}$ for wt APE1 and 0.001 s$^{-1}$ for APE1-H309N indicates that the chemical step for APE1-H309N is at least a factor of $10^5$ faster for wt APE1 than for APE1-H309N. The effect of the D210A mutations is even greater because the reaction it catalyzes is even slower.

Although these observations in and of themselves may not shed light on the exact function of His-309 and Asp-210 during catalysis, they do show that both residues are absolutely critical for efficient chemistry. Data from NMR studies indicates that His-309 is protonated and at a distance in the crystal structure to be hydrogen bonded to one of the non-bridging oxygens in the phosphoryl group that will be hydrolyzed (16,19). This residue may serve to stabilize some of the negative charge that develops on this oxygen in the transition state. The kinetic data obtained in this work are in concordance with a key role such as this in catalysis. Likewise, the severe effect of mutating Asp-210 to Ala suggest that it plays a critical role in catalysis. Other groups have made different mutations to Asp-210 and all have a dramatic effect on the AP endonuclease reaction with two notable exceptions. First, APE1-D210H was 15-20-fold more active than other mutants leading to the proposal that Asp-210 is donating a proton to the leaving group which can be accomplished by the His substitution (21). Similarly, mutation of Asp-210 to Glu reduced the steady-state AP endonuclease activity by 500-fold without changing $K_m$ suggesting that the acidic nature of the residue at this position is critical and that Asp-210 is required for chemistry but not substrate binding (22).

**Implications for the base excision repair pathway**— Human APE1 is essential for normal growth and development as well as cell survival. One of the key functions of APE1 is processing AP sites that form spontaneously and as the result of the first step in the BER pathway catalyzed by DNA glycosylases. In either case, the nicked AP product formed by APE1 must be repaired by downstream enzymes in the BER pathway. Coordination of the enzymatic activities in BER is essential to ensure that once repair is initiated, it is completed. Both the substrates (AP sites) and products (single-strand breaks) of APE1 are cytotoxic if they accumulate in the genome. This means that APE1 must bind and process AP sites as quickly as they accumulate in the genome and not generate single-strand breaks faster than they can be processed. It has been estimated that as many as 2,000 - 10,000 depurination events that generate AP sites occur per cell per day (32). The ability of APE1 to bind and incise AP sites at a rate that is limited by diffusion and its abundance in the cell suggests that it can keep up with this spontaneous mutation load to efficiently process the AP sites that are formed. With the exception of uracil DNA glycosylase, the activities of DNA glycosylases are several orders of magnitude slower than the AP endonuclease activity of APE1 suggesting that the relative kinetics of formation and processing of AP sites during BER coordinates these first two steps so that AP sites are not made faster than they can be processed (24,33-39). After incising AP sites, APE1 is slow to dissociate from the product. This slow dissociation may allow for the coordination of AP endonuclease activity with the dRP lyase and DNA polymerase activities of DNA polymerase β or strand-displacement activities of other DNA polymerases. APE1 may remain bound to product to recruit DNA polymerase β (40,41) or may simply remain bound so that free nicked AP sites do not accumulate at a rate faster than they can be “found” by a DNA polymerase.
REFERENCES


FOOTNOTES

1This work was supported by a grant from the National Institutes of Health (GM055596) to L.B.B.
2Abbreviations: AP, apurinic, apyrimidinic, or abasic; APE1, AP endonuclease 1; BER, base excision repair; RhX, X-rhodamine; wt, wild-type.

FIGURE LEGENDS

Fig. 1. Binding of wild-type APE1 to AP-DNA. Wild-type APE1 was added to 20 nM (filled circles) or 40 nM (filled triangles) RhX-labeled AP-DNA in assay buffer supplemented with EDTA to inhibit the AP endonuclease reaction. Average anisotropy values for RhX calculated from polarized intensities are plotted as a function of APE1 concentration. Data from 4 independent experiments were globally fit to Equation 3 (solid lines) to calculate an equilibrium dissociation constant ($K_d$) of 11 +/- 2 nM. Average values are shown here, error bars indicate standard deviation. Anisotropy values for free DNA, 20 nM (open circles) and 40 nM (open triangles), before addition of APE1 are also shown.

Fig. 2. Pre-steady-state AP endonuclease kinetics under multiple-turnover conditions. Incision 5’ to AP sites was measured in reactions containing 40 nM wt APE1 (upper panel) and 80 (circles), 160 (squares), 250 (triangles), 350 (inverted triangles), or 500 nM (diamonds) AP-DNA or 200 nM wt APE1 (lower panel) and 500 (circles), 1000 (squares), or 2000 nM (triangles) AP-DNA. Solid lines through the data are the result of a fit to Equation 5. Time courses for the AP endonuclease reactions containing 200 nM APE1 (lower panel) were repeated, and average values with standard deviations (error bars) of the amount of product formed at each time point are shown. Reactions containing 500 nM AP-DNA were done in duplicate and those containing 1000 and 2000 nM AP-DNA were done in triplicate. A few of the individual time points (no error bars shown) are the result of a single measurement. Average values with standard deviations calculated for burst rates were 253 ± 62, 348 ± 15, and 727 ± 239 s$^{-1}$ for reactions containing 500, 1000, and 2000 nM DNA, respectively, and 200 nM APE1.

Fig. 3. Stoichiometric binding of wt APE1 to AP-DNA. Binding of wt APE1 to RhX-labeled AP-DNA was measured in anisotropy assays that contained a high concentration of DNA (1000 nM) relative to the $K_d$ for DNA binding (11 nM) determined in Fig. 1. Calculated anisotropy values are plotted as a function of APE1 concentration (filled circles) and fit (solid lines) by a modified version of Equation 3. In the modified equation, the term representing the concentration of APE1, Et, was multiplied by a factor, a, that represents the fraction of active enzyme which was calculated from the fit to be 1.1 +/- 0.2. Empty circles show the anisotropy of AP-DNA prior to addition of APE1. Average data from 2 independent experiments are shown and error bars indicate standard deviation.
Fig. 4. Pre-steady-state kinetics of AP site incision under single-turnover conditions. AP endonuclease reactions contained 0.02 µM AP-DNA and concentrations of wt APE1 ranging from 0.04 – 6 µM as indicated in the figure. Solid lines result from fitting these time courses to a double exponential rise (Equation 6). The lower panel shows the same 8 time courses on an expanded time scale. Time courses at concentrations of 0.04 (filled circles), 0.08 (filled squares), 0.5 (filled inverted triangles), and 1 (open circles) µM APE1 were done in duplicate and average values with standard deviations (error bars) of the experiments are shown. In some cases, the error bars are physically smaller than the symbol used to represent the data point, however, data points at 0.002 s and 1.5 s represent a single measurement for the 0.04 µM APE1 reaction. Observed rate constants for each experiment were calculated using Equation 6 and average values with standard deviations were calculated for duplicate experiments. Average values for the observed rates of the rapid phase were 23 ± 5, 77 ± 3, 232 ± 37, and 441 ± 40 s⁻¹ for the 0.04, 0.08, 0.5 and 1 µM APE1 reactions, respectively, and average values for the observed rates of the slow phase were 4.3 ± 0.1, 13 ± 1, 19 ± 6, and 23 ± 8 s⁻¹ the 0.04, 0.08, 0.5 and 1 µM APE1 reactions, respectively.

Fig. 5. Plot of observed rate constants from fits of single-turnover data as a function of wt APE1 concentration. Observed rate constants calculated for the rapid phase of single-turnover data plotted in Fig. 4 and Supp. Fig. 1 were plotted as a function of APE1 concentration and fit (solid line) to a line. At concentrations greater than 2 µM APE1 accurate values for this observed rate constant could not be determined because the reactions were too fast. An apparent binding constant (k_{on,app}) of 3.5 x 10⁸ M⁻¹ s⁻¹ and apparent dissociation constant (k_{off,app}) of 47 s⁻¹ were calculated from the slope and y-intercept, respectively.

Fig. 6. DNA binding and AP endonuclease activities of APE1-H309N. A, Plots show the increase in anisotropy when APE1-H309N binds RhX-labeled AP-DNA. Binding assays contained either 20 nM (circles) or 40 nM (triangles) AP-DNA. Open symbols show anisotropy values for free DNA before addition of APE1-H309N. Anisotropy data were fit (solid curves) to Equation 3 and plotted as in Fig. 1 to determine the equilibrium dissociation constant (K_d) of 53 +/- 7 nM. B, Plots show single-turnover kinetics of AP site incision catalyzed by APE1-H309N. AP endonuclease assays contained 20 nM AP-DNA and 100, 200, 600, and 1000 nM APE1-H309N. Time courses for product formation were fit (solid curves) to a single exponential rise (Equation 7).

Fig. 7. AP-DNA binding activity of APE1-D210A. A, Binding was measured by measuring the increase in anisotropy when APE1-D210A bound RhX-labeled AP-DNA (20 nM). Anisotropy values were calculated for free DNA (open circles) and after addition of APE1-D210A (filled circles). The anisotropy values after APE1 addition from 2 independent experiments were fit by Equation 3 to determine the equilibrium dissociation constant (K_d) of 2.4 +/- 1.3 nM. B, DNA binding was measured using 32P-labeled DNA in an electrophoretic mobility shift assay. The concentration of protein-DNA complex formed when APE1-D210A was added to 5 nM AP-DNA is plotted as a function of APE1-D210A concentration. Binding data was fit (solid lines) to Equation 4 to determine a dissociation constant (K_d) of 1.5 +/- 0.4 nM.
Fig. 1

APE1 (nM) vs. Anisotropy for wt APE1 and RhX

APE1 (nM)

Anisotropy
AP-DNA concentration varied

wt APE1

\[ \begin{align*}
\text{incised product (nM)} & \quad \text{time (s)} \\
0 & \quad 0 \\
20 & \quad 0.5 \\
40 & \quad 1 \\
60 & \quad 1.5 \\
80 & \quad 2 \\
\end{align*} \]

40 nM APE1

\[ \begin{align*}
\text{incised product (nM)} & \quad \text{time (s)} \\
0 & \quad 0 \\
20 & \quad 0.02 \\
40 & \quad 0.04 \\
60 & \quad 0.06 \\
80 & \quad 0.08 \\
100 & \quad 0.1 \\
\end{align*} \]

200 nM APE1
wt APE1

\[ ^{32}\text{P} \rightarrow ^{32}\text{P-OH} \]

![Graph showing the incised product (nM) over time (s) for wt APE1 at different concentrations (0.04 µM to 6 µM).](image)

**Incised product (nM)**

0 0.01 0.02 0.03 0.04 0.05 0.06

\[ 0 \quad 0.2 \quad 0.4 \quad 0.6 \quad 0.8 \quad 1 \quad 1.2 \quad 1.4 \quad 1.6 \]

time (s)

**Expanded time scale**

![Graph showing the incised product (nM) over time (s) for wt APE1 at different concentrations (0.04 µM to 6 µM) on an expanded time scale.](image)

**Incised product (nM)**

0 5 10 15 20

\[ 0 \quad 0.01 \quad 0.02 \quad 0.03 \quad 0.04 \quad 0.05 \quad 0.06 \]

time (s)
Fig. 6.

A

APE1-H309N

RhX

RhX

0.27

0.26

0.25

0.24

0.23

0.22

0.21

0.2

APE1-H309N (nM)

APE1-H309N (nM)

B

APE1-H309N

$^{32}$P

$^{32}$P

OH

$^{32}$P

product (nM)

product (nM)

time (s)

time (s)
Pre-steady-state kinetic characterization of the AP endonuclease activity of human AP endonuclease 1
Robyn L. Maher and Linda B. Bloom

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