Human Apurinic/Apyrimidinic Endonuclease Is Processive[†,‡]

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ABSTRACT: Apurinic/apyrimidinic endonuclease (AP endo) is believed to play a critical role in repair of oxidative damage of DNA and is proposed to initiate repair of most abasic sites in the base excision repair pathway. AP endo makes a single nick 5′ to an abasic site in double-stranded DNA. In this study, we investigated whether AP endo locates an abasic site through a processive or a distributive mechanism. We used a linear multi-abasic site substrate (concatemer), synthesized by ligating together identical 25-nucleotide monomeric units (25-mers). We first determined that the 25-mer monomer from which the concatemers were prepared was nicked by AP endo in a fashion similar to that of the previously published 49-mer substrate with a different sequence. Steady state parameters K_m and k_{cat} and single-turnover parameters for substrate binding were comparable to previously published values. Using the multi-abasic site concatemer, we demonstrated that AP endo was capable of cleaving approximately seven to eight abasic sites, traveling at least 200 nucleotides, before dissociating from its substrate. Thus, AP endo, like uracil DNA glycosylase, behaves in a quasi processive fashion. Processivity could be separated from catalysis, since processivity was maximal at 25 mM NaCl, while the rate of cleavage was maximal at 125 mM salt. In short, nicking activity was maximized close to physiological salt molarities while processivity was midrange at physiological salt concentrations. The latter is likely to be subject to tight regulation by small changes in ionic strength.

Under normal physiological conditions, about 10 000 abasic sites are generated per mammalian genome per day (*1*, *2*). These are repaired rapidly, since steady state levels of abasic sites are generally low (*2*). As in any metabolic pathway, efficiency depends on recognition of the abasic site and the enzymology and regulation of the individual steps in the repair pathway.

One major lesion in DNA that generates abasic sites is the presence of uracil, which arises either via deamination of cytosine or through the misincorporation of uracil during replication (*3*). The first enzyme to recognize uracil, uracil DNA glycosylase (UDG),[1] scans DNA in a processive fashion (*4*−*6*). The enzyme causes a pinch in the DNA backbone that forces the mispaired uracil to flip out of the major groove (*7*−*9*). Despite its behavior on a multi-uracil containing substrate, UDG has a high turnover number on a substrate with a single uracil (*10*, *11*). Another major source of abasic sites is spontaneous depurination, which occurs in the absence of enzymatic intervention (*3*). The primary enzyme responsible for initiating repair of abasic sites is apurinic/apyrimidinic endonuclease (AP endo, also known as APE, APEX, HAP1, and Ref1) (EC 3.1.25.2) (*12*−*18*, *41*). AP endo cleaves the abasic site 5′ to the phosphodeoxyribose, thus generating the free 3′-hydroxyl required for a DNA polymerase to fill in the resulting gap (*19*−*22*). Although the enzyme cleaves only the strand containing the abasic site, cleavage requires the presence of the intact complementary strand. Despite the fact that the enzyme is ubiquitously expressed, it is preferentially localized in different subcellular compartments (*23*), and its synthesis is selectively activated by nontoxic levels of reactive oxygen species (*24*).

How does AP endo recognize its abasic site substrate to maintain a high repair efficiency? AP endo is comprised of 319 amino acids with a molecular mass of 35.5 kDa (*25*). The enzyme in solution consists of a disordered amino terminus up to residue 36 and a highly ordered globular domain, as shown by domain mapping experiments (*26*). X-ray crystallography of the enzyme lacking the amino terminus also shows the globular nature of the bulk of the molecule (*27*). Like UDG, human AP endo has a high turnover number when there is a single abasic site at position 21 of a 49-mer oligomer (10 s^{-1}) and binds efficiently (K_d = 0.8 nM) to its substrate (*21*, *28*).

We present evidence here that when there are multiple abasic sites as part of a linear concatemer, AP endo behaves in a quasi processive fashion much like UDG. Since abasic sites can arise either enzymatically or spontaneously, it is critical that AP endo and the glycosylases be able to scan the genome efficiently and independently. Thus, recognition of the initial lesion and entry into the repair pathway from two distinct sources can be maximized with minimal interference from each other.

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[1] Abbreviations: AP endo, apurinic/apyrimidinic endonuclease; DAB buffer, 30 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, and 5% glycerol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HDP, heat degradation product, the β-elimination product of an 11-mer oligonucleotide containing an abasic site (see ref *21*); PNK, T4 polynucleotide kinase; UDG, uracil DNA glycosylase.

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EXPERIMENTAL PROCEDURES

The oligonucleotides (U-25-mer) 5′-GGG GCT CGT A**U**A AGG AAT TCG TAC C-3′ containing a uracil at position 11 and the complementary strand (G-25-mer) 5′-CCC CGG TAC GAA TTC CTT GTA CGA G-3′, initially designed for examining UDG processivity (*4*), were synthesized and gel purified by Genosys Biotechnologies (The Woodlands, TX). Preparation of the abasic site-containing substrate is described for monomeric and concatemeric substrates in the appropriate sections.

Enzyme. Expression and purification of human AP endo from pXC53 are described in detail in a previous publication (*21*). As described at that time, the concentration was determined by dye binding assay, calibrated by amino acid analysis (*21*). Enzyme, stored at −80 °C, was diluted immediately before use to the appropriate concentration in ice-cold dilution buffer consisting of 50% glycerol in 50 mM Hepes/NaOH (pH 7.4), 0.1 mM EDTA, and 125 mM NaCl. Diluent contributed 25 mM NaCl to the final salt concentration in each reaction described below.

Studies with the Monomeric Substrate. Throughout this study, "monomer" refers to a single 25-mer unit with the structure written above, while concatemer refers to double-stranded monomers which have been ligated together as described below. The U-25-mer (20 nmol) was labeled at the 5′-end by means of T4 polynucleotide kinase (PNK) (U.S. Biochemicals, Cleveland, OH) and [γ-^{32}P]ATP (6000 Ci/mmol, Dupont/NEN, Boston, MA), annealed to its complement, and separated from unincorporated [γ-^{32}P]ATP as described in ref *21*.

Preparation of the Monomeric Substrate for Kinetic Assays. Abasic sites were created by using *Escherichia coli* UDG (Ung, Epicentre Technologies, Madison, WI; 1 unit/100 pmol of uracil residues) as described previously (*21*). The reducing agent $NaBH_4$ (0.1 mM unless otherwise noted) was added during the incubation with UDG to minimize the formation of β-elimination product. UDG treatment was terminated by heating the reaction mixture to 70 °C for 5 min, followed by slow cooling to allow for reannealing of DNA strands.

Kinetic Assays with the Monomeric Substrate. All assays were performed at room temperature (22 °C) in a reaction volume of 5 μL containing Hepes/NaOH buffer (pH 7.4), 0.1 mM EDTA, and 5 mM $MgCl_2$ with the indicated final NaCl concentration. Reactions were terminated by the addition of 1 μL of 0.5 M EDTA to a final concentration of 87 mM. The substrate and product were resolved by means of gel electrophoresis using 20% denaturing polyacrylamide gels containing 8 M urea. Product formation was quantitated by means of PhosphorImager analysis using the Molecular Dynamics Storm 840 PhosphorImager (Sunnyvale, CA) and ImageQuant software.

Steady state experiments in which the relationship of concentration versus velocity was examined were performed over a substrate range of 16−300 nM and with an enzyme concentration of 0.2 nM. K_m and V_{max} values were obtained from linear regression analysis using individual duplicate data points from three independent experiments. The standard error for $1/V_{max}$ was obtained from regression analysis. The standard error in the x-intercept ($-1/K_m$) was approximated by the first term in the Taylor expansion as $\delta x = -(\delta b)/m + (\delta m)b/m^2$, where b is the y-intercept and m is the slope of the line. The error in K_m was estimated as K_m^2 multiplied by δx. Similarly, the error in V_{max} was estimated as V_{max}^2 multiplied by the error in $1/V_{max}$.

Single-turnover assays for obtaining substrate association (k_{on}) and dissociation (k_{off}) parameters were performed using a trap to prevent the enzyme from undergoing more than one round of catalysis (*21*). Trap consisted of 2 mg/mL heparin and 21 μM HDP. AP endo (0.4 nM) and substrate (4 nM) were incubated for various times ranging from 5 to 30 s in the presence of 4 mM EDTA without additional Mg^{2+}. At the end of each time point, 10 mM Mg^{2+} and trap were added for an additional 20 s, giving a final reaction volume of 5 μL. The reaction was terminated after 20 s by the addition of 1 μL of 0.5 M EDTA. To calculate the substrate binding and dissociation constant, a simulated binding curve was produced using HopKINSIM (*29*).

Studies with the Concatemeric Substrate. The production of the concatemeric substrate was based on methodology described by Bennett et al. (*4*). The phosphorylation reaction mixture (550 μL) contained 20 nmol of U-25-mer, 1.0 mCi of [γ-^{32}P]ATP (6000 Ci/mmol), 200 units of T4 PNK in 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, and 0.1 mM EDTA. The mixture was incubated at 37 °C for 15 min, at which time ATP was added to a final concentration of 4 mM. The labeling reaction was stopped after 60 min by adjusting the mixture to 10 mM EDTA. The labeled U-25-mer (5 nmol) was then annealed to an equal amount of G-25-mer by heating for 2 min at 85 °C, 15 min at 65 °C, 15 min at 37 °C, 15 min at 25 °C, and finally 15 min on ice. The annealed product was ligated in a 675 μL reaction mixture containing 5 nmol of annealed DNA, DAB buffer [30 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, and 5% glycerol], 5 mM $MgCl_2$, 1 mM ATP, 100 units of PNK, and 14 units of T4 DNA ligase (Gibco BRL, Gaithersburg, MD). The ligation mixture was incubated for 2 h at 16 °C, then supplemented with 360 nmol of ATP and 14 units of DNA ligase, and incubated overnight at 16 °C. After the ligation mixture was passed over a Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ) gel filtration column (1.82 cm^2 × 47 cm), previously equilibrated with Hepes buffer [50 mM Hepes/NaOH (pH 7.4) and 0.1 mM EDTA], fractions of 600 μL were collected. Ligated products in each fraction were resolved on a 4% denaturing polyacrylamide gel containing 8 M urea and electrophoresed until the dye front migrated approximately 30 cm. Size markers consisted of a DNA ladder between 25 and 2652 bp (Gibco BRL) which had been labeled at the 5′-terminus. The first fraction eluting from the column with high-molecular mass DNA was discarded because the sizes of the bands were offset from those in fractions that followed. Otherwise, samples containing ≥250 bp were pooled and concentrated to a final concentration of 5 μM in 25-mer monomers using centrifugal concentrators with a 10 kDa pore size (Pall Filtron, Northborough, MA). The amount of 25-mer monomers recovered in the pooled fractions was calculated by first obtaining the ratio of the amount of ^{32}P in the pooled fractions to the total amount of ^{32}P recovered from the column and then multiplying the ratio by the amount of 25-mer monomer in concatemers applied to the column. The average size of concatemers in the pooled fractions was 350 bp. Thirty-four percent of

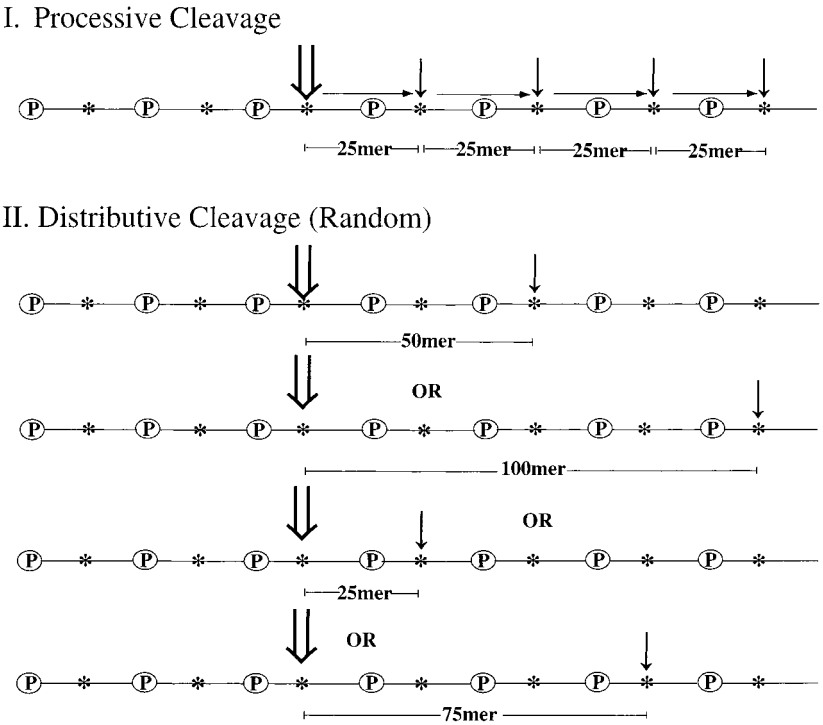

FIGURE 1: Schematic diagram of hypothetical results of cleavage of a concatemeric substrate by AP endonuclease. The figure depicts two possible scenarios for cleavage, (I) a processive manner of cleavage and (II) a distributive mode of cleavage. Although the DNA is double-stranded, only the U-containing strand is shown. The cartoon makes no assumptions about whether the enzyme initially binds to double-stranded DNA or to a DNA end and tracks to an abasic site or whether it binds to an abasic site directly. Further, the processive movement of AP endo is presented in a unidirectional fashion for the sake of simplicity, although processivity is likely to be a random diffusion process without a directional component. Radiolabeled 5′-ends of monomer in the U-containing strand are represented by Ⓟ. The asterisks represent the locations of uracils in each 25-mer and, after treatment with UDG, the locations of abasic sites. AP endo's initial nick is depicted by the ⇊, and subsequent nicks are represented by ↓.

the concatemers were ≥400 bp; 56% were ≥300 bp, and 92% were ≥125 bp.

Abasic sites for the multimeric substrate were created as previously described for the monomeric substrate with the exception being that the $NaBH_4$ concentration was reduced from 0.1 to 0.05 mM. This concentration of $NaBH_4$ was enough to stabilize the substrate (data not shown) and contributed half as much Na^+ to the reaction mix.

Processivity Experiments. The action of AP endo on its multimeric substrate was measured in final reaction mixtures containing concatemeric substrate (1.65 μM in monomers) and 0.01 nM AP endo. The low enzyme:substrate ratio maximized the likelihood that no more than one enzyme molecule was associated with any individual concatemer molecule at any time. Preliminary experiments showed that at this enzyme and substrate concentration less than 30% of the substrate was consumed during a 90 min interval. Processivity experiments were performed at room temperature for 0−90 min. Final reaction mixtures (5 μL) contained 5 mM $MgCl_2$ and Hepes buffer in addition to NaCl, substrate, and enzyme as indicated. The reactions were initiated by the addition of AP endo and terminated by the addition of 1.0 μL of 0.5 M EDTA (pH 8).

RESULTS

To determine whether AP endo behaved in a processive or distributive fashion, cleavage experiments were performed using a concatemeric substrate containing an abasic site after every 25th nucleotide. Figure 1 depicts the structure of the concatemer and the possible products. Enzyme is expected to bind to DNA in a random fashion anywhere along the concatemer and to track to an abasic site where it performs the first round of catalysis. Thus, product at the earliest time intervals will have a random size distribution regardless of how the enzyme proceeds thereafter. After the first round of catalysis, the size distribution of products will continue to be random if the enzyme dissociates from the product after every round of catalysis so that the process is distributive. On the other hand, if the process is processive, second and subsequent cleavages will be one 25-mer monomer away from the initial cleavage site and the 25-mer monomer will accumulate until the enzyme dissociates from DNA. This model makes no assumptions about the direction in which the enzyme tracks but stipulates only that the enzyme cannot track across an abasic site without cleaving it.

(A) 25-mer Monomer

Before processivity experiments could be conducted on the concatemeric substrate, it was necessary to determine how AP endo acted on the 25-mer monomer. In particular, did the enzyme nick the 25-mer monomer in a fashion kinetically similar to that of the previously published 49-mer substrate with an abasic site at position 21 (*21*)?

Time Course of Cleavage of the Monomeric Substrate. A time course experiment between 0 and 60 s was performed on the monomeric substrate (Figure 2). The initial substrate and enzyme concentrations for the time course were 250 and 0.1 nM, respectively, while the Na^+ concentration was 50 mM. The time course proved to be linear for the extent of the experiment, and no more than 40% of the substrate was

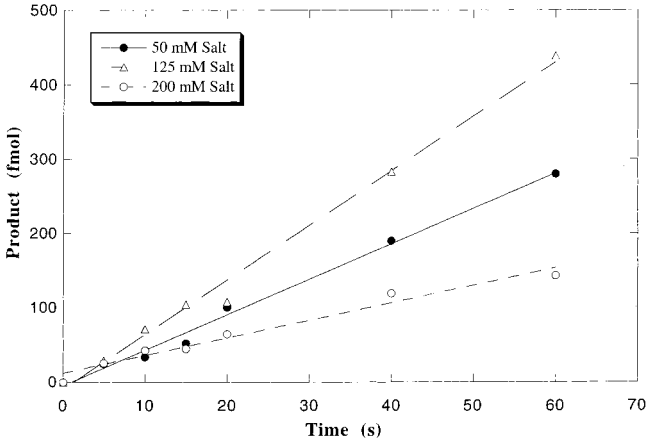

FIGURE 2: Time course of nicking by AP endo acting on a 25-mer monomeric substrate. The time dependence was examined at salt concentrations of 50, 125, and 200 mM between 0 and 60 s. The initial substrate and AP endo concentrations were 250 and 0.1 nM, respectively. These data are from a representative experiment.

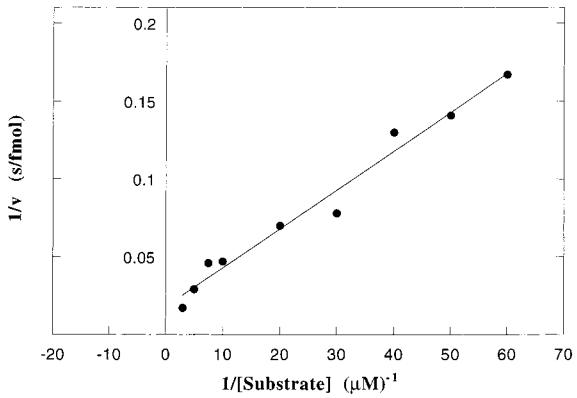

FIGURE 3: Concentration dependence of nicking of a monomeric substrate. The steady state concentration dependence for the monomer was examined over 10 s time intervals with a substrate concentration range of 16−300 nM and an enzyme concentration of 0.2 nM. Each measurement was made in duplicate. The data presented in this Lineweaver−Burk plot are the average of three independent experiments. The K_m, calculated by analysis of covariance from three independent experiments, was 120 nM, while the k_{cat} was 6 s^{-1}.

exhausted after 60 s (Figure 2). Since salt sensitivity is a hallmark of processivity, it was important to observe the salt sensitivity for cleavage of the 25-mer monomer on which processivity cannot occur. The time dependence was examined on the 25-mer monomeric substrate at two additional Na^+ concentrations (125 and 200 mM). Again, the rate of nicking was linear for the extent of the experiment. At 125 mM salt, the rate of nicking was about twice that at 50 mM salt and four times that at 200 mM salt. All subsequent experiments with the 25-mer monomeric substrate were performed at a total salt concentration of 50 mM so that they would be comparable with previously published reports (*21*, *28*).

Steady State Kinetics of Cleavage of the Monomer. Figure 3 depicts the concentration dependence of AP endo at 50 mM Na^+, which was used to determine both K_m and k_{cat} for the 25-mer. The K_m for the 25-mer monomeric substrate was calculated from the Lineweaver−Burk plot to be 120 ± 50 nM (standard error, $n = 3$), which agrees well with the observed K_m for the 49-mer published by Strauss et al. (*21*)

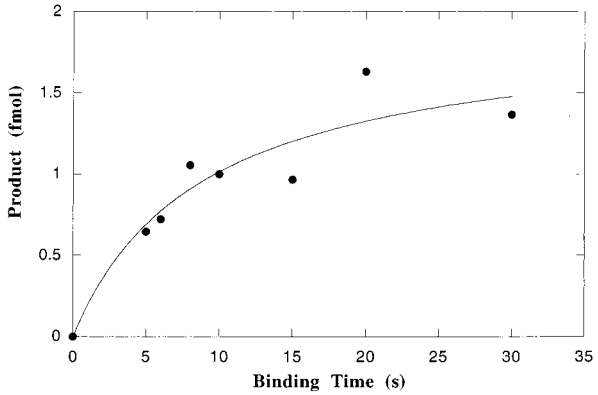

FIGURE 4: Binding curve for the 25-mer monomeric substrate under single-turnover conditions. After AP endo (0.4 nM) and 25-mer containing an abasic site (4 nM) were incubated in the absence of Mg^{2+} for 0−30 s, the ES concentration was determined at each time point by initiating the reaction with the simultaneous addition of Mg^{2+} (10 mM) and trap (2 mg/mL heparin and 21 μM HDP). Reactions were terminated after 20 s by the addition of EDTA as described in Experimental Procedures. These data are the average of four independent experiments.

of 100 nM. The k_{cat} value was 6 ± 0.3 s^{-1}, which is comparable to the value of 10 s^{-1} previously reported (*21*). The catalytic efficiency for the 25-mer monomer (k_{cat}/V_{max}) was calculated to be 0.5 × 10^8 M^{-1} s^{-1}, which implies that this reaction is diffusion-limited (*30*), as is the reaction for the 49-mer.

Single-Turnover Kinetics for Cleavage of the Monomer. Figure 4 represents results of a single-turnover study with the 25-mer monomer. AP endo (0.4 mM) was allowed to bind to 25-mer (4.0 nM) in the absence of Mg^{2+} for the indicated periods of time. The concentration of the ES complex was determined at each time point by initiating a single round of enzymatic catalysis with the simultaneous addition of Mg^{2+} and trap. The experimental binding curve produced an apparent half-time ($t_{1/2}$) of 6 s, an $[ES]_e$ of 0.28 nM, and a K_d of 1.2 nM. By kinetic simulation (*21*, *29*), the constants for substrate binding (k_{on}) and dissociation (k_{off}) were estimated to be 2 × 10^7 M^{-1} s^{-1} and 0.03 s^{-1}, respectively, to be compared with previously reported values of 5 × 10^7 M^{-1} s^{-1} and 0.04 s^{-1}, respectively (*21*). Therefore, AP endo exhibited very similar kinetics with the 25-mer substrate containing a single abasic site compared with the published data on the 49-mer containing a single abasic site within a different sequence context.

(B) Concatemer

Time Course of Cleavage of the Concatemeric Substrate. An initial time course experiment at 50 mM Na^+ demonstrated that the rates of appearance of 25-mer, 50-mer, and 75-mer products were linear for the duration of the experiment (panels a and b of Figure 5). The amount of 25-mer well exceeded the amount of 50-mer or 75-mer, even at 10 min, while after 20 min, about 80% of the product consisted of 25-mer.

Ratios of Cleavage. We hypothesized that the relative amounts of 25-mer and 50-mer products could be used as a measure of processivity. A ratio of 1 would indicate random appearance of both 25-mer and 50-mer, e.g., a distributive mechanism with random cleavage of substrate. On the other hand, a ratio of >1 would reflect nonrandom, processive

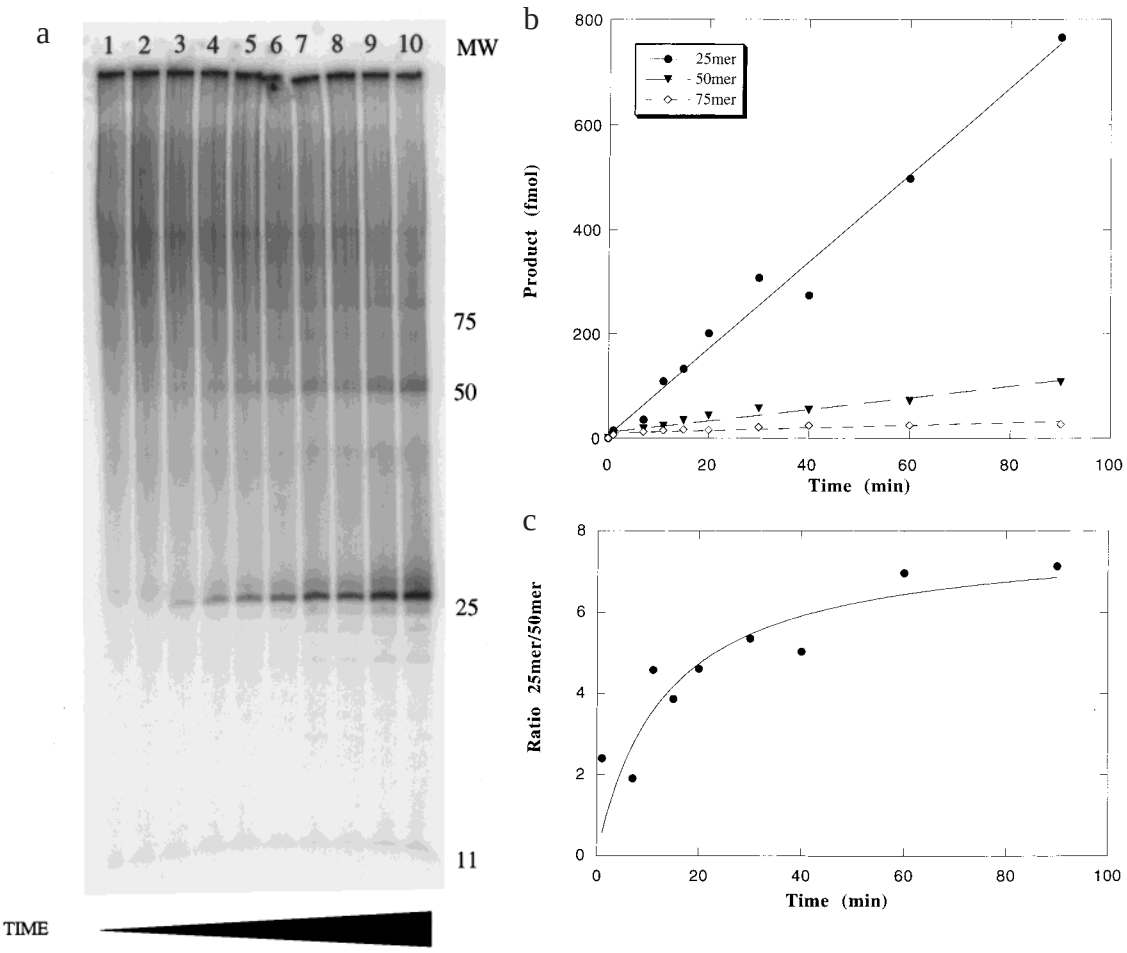

FIGURE 5: (a) Cleavage of the concatemeric substrate by AP endo. Nicking reactions were performed at a substrate concentration of 1.65 μM in 25-mer monomers and an AP endo concentration of 0.01 nM for time intervals between 0 and 90 min. The total Na^+ concentration in this experiment was maintained at 50 mM. The substrate and product were resolved by denaturing gel electrophoresis as described in Experimental Procedures. Time points from left to right are 0 (no enzyme), 1, 7, 11, 15, 20, 30, 40, 60, and 90 min. Bands accumulating at the top were concatemers that were too large to be resolved on the 20% gel used to quantitate smaller oligonucleotides. The distribution of concatemers at time 0 represents the size distribution of substrate molecules before addition of enzyme. (b) Quantitation of the appearance of products of different size classes. These data are the average of two independent experiments, including the one whose results are shown in panel a. (c) Ratio of cleavage products of different size classes [25-mer/(50-mer + 75-mer)] obtained from the concatemeric substrate. Data are taken from the experimental results presented in panel b.

cleavage. Further, we hypothesized that the magnitude of the ratio would indicate how many cleavages AP endo makes, before it dissociates from the concatemer. If the enzyme were fully processive and unable to dissociate from the concatemer until all abasic sites were cleaved, then the steady state ratio would reflect the size distribution of concatemers. Since the median size of concatemers was 350 bp, the predicted ratio would be approximately 13:1.[2] Figure 5c depicts the ratios of 25-mer to 50-mer + 75-mer for cleavage of the concatemeric substrate over the 90 min time span shown in Figure 5a. Since the amounts of 75-mer cleavage products were too small to affect the ratios, they were omitted from subsequent calculations. The ratios obtained at the earliest times (about 2:1) increased until they reached a steady state between 7:1 and 8:1 at 60 min. Since only one AP endo molecule was likely to be in contact with each DNA molecule and since there was one abasic site located after every 25th nucleotide, we inferred that AP endo was capable of cleaving on average seven or eight abasic sites, before it dissociated from the DNA molecule. Therefore, under these conditions, AP endo was able to traverse the DNA cleaving an abasic site after every 25th nucleotide for a minimum distance of approximately 175−200 nucleotides. This distance is a minimum, because processive movement is likely to be random and because of potential end effects that might occur when the enzyme cleaves the site nearest the 5′- or 3′-end of the concatemer.

Salt Dependence for Nicking of the Concatemer. Processive enzymes are usually highly salt sensitive (*31*). The time dependence of nicking the concatemer at three different Na^+ concentrations of 50, 125, and 200 mM was examined (Figure 6). The experiments were similar to the processivity experiments described above, with a substrate concentration of 1.65 μM, an AP endo concentration of 0.01 nM, and time intervals from 0 to 90 min. The rate of product formation was again linear over the course of the experiment. The pattern was the same as the monomeric salt dependence displayed in Figure 2: the rate of product formation was maximal at 125 mM Na^+. At 50 mM Na^+, the rate was about

[2] The predicted ratio of 25-mer monomers to 50-mers for full processivity was calculated as ([25-mer monomers]/[50-mer]) − 1, since the ultimate cleavage at the 5′-end of the concatemer yields an 11-mer. We are unable to detect the 14-mer which is the 3′-product of the ultimate cleavage at the 3′-end, because it contains no labeled phosphate.

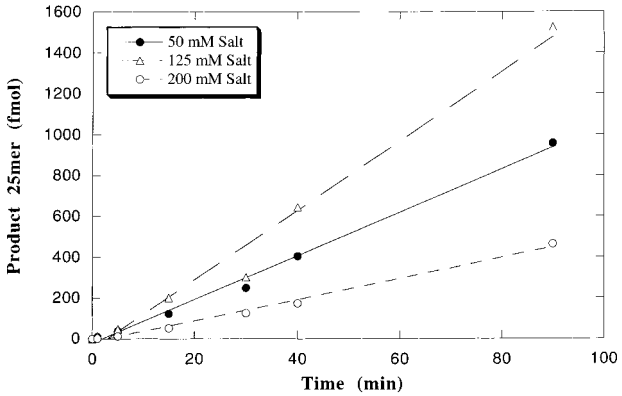

FIGURE 6: Time dependence of nicking of the concatemer at different salt concentrations. The amounts of cleavage products at three different salt concentrations (50, 125, and 200 mM) as a function of time are shown. These data are the average of two independent experiments.

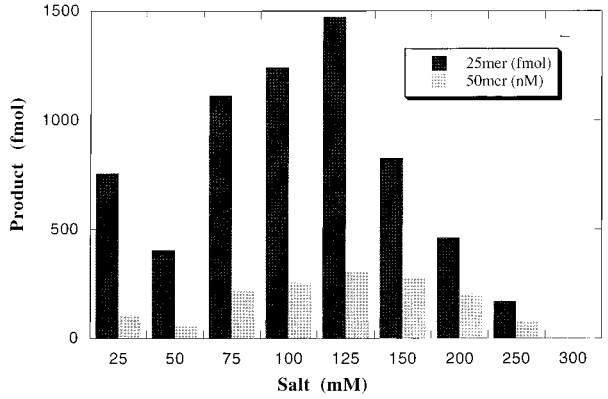

FIGURE 7: Changes in the amounts of different product classes with increasing salt concentrations. A NaCl concentration range from 25 to 300 mM was utilized. The amounts of both 25-mer and 50-mer products were determined at 90 min time points for each salt concentration. Substrate and enzyme concentrations were held constant at 1.65 μM and 0.01 nM, respectively. These data are an average of two independent experiments.

half-maximal, while at 200 mM Na^+, the rate of product formation was about 25% maximal. Since the concentration of the substrate was 1.65 μM in 25-mer monomers and the enzyme concentration was 0.01 nM, chosen to ensure that the enzyme was fully saturated, the measured velocities were likely to be maximal. Consequently, we calculated a k_{cat} from the data presented in Figure 6. Steady state k_{cat} values were 1, 3, and 6 s^{-1} at 200, 50, and 125 mM NaCl, respectively. These values are lower bound estimates because they are derived from the rate of appearance of 25-mer and do not include total nicking activity of other size classes. Therefore, the turnover numbers for 25-mer monomer and concatemer substrates under these conditions were comparable.

To define more precisely the effects of salt on AP endo's ability to perform processively, a salt titration between 25 and 300 mM NaCl was performed (Figures 7 and 8). Both 25-mer and 50-mer products were quantitated at 90 min time points for each salt concentration. Substrate and enzyme were held constant at concentrations of 1.65 μM and 0.01 nM, respectively. The effects of salt on nicking and processivity were strikingly different. As seen in Figure 6, the total amount of 25-mer and 50-mer product increased with increasing salt concentration up to 125 mM Na^+, and then the amount of product decreased at salt concentrations above

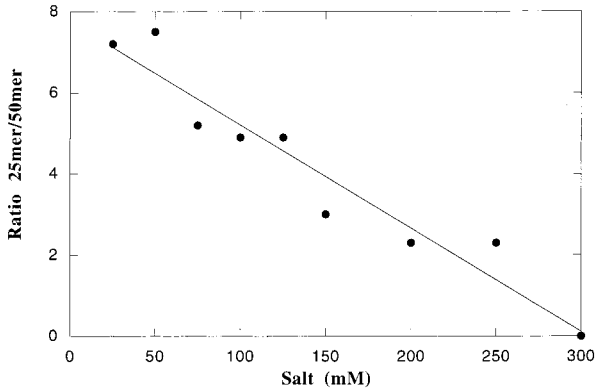

FIGURE 8: Decrease in the degree of processivity with an increase in Na^+ concentration. Ratios of 25-mer product to 50-mer product determined from the salt titration described in Figure 7 reveal a linear decrease in the degree of processivity with increasing salt concentrations.

125 mM Na^+ until no product could be detected at 300 mM salt. In striking contrast, monovalent cation added to a concentration above 50 mM decreased the level of processivity, since a linear decrease in ratios of 25-mer to 50-mer occurred with increasing Na^+ concentrations (Figure 8). The ratios of 25-mer to 50-mer dropped from 8:1 at 50 mM salt to about 5:1 at 125 mM and 2:1 at 200 mM salt. The decrease in the ratio, resulting from fewer "nickings", was completely consistent with the conclusion that AP endo is a quasi processive enzyme, and that processivity arises through ionic interactions between DNA and enzyme (see below for further discussion). Where Figure 7 displays maximal nicking by AP endo on the concatemer at 125 mM NaCl, Figure 8 displays a 30% decrease in the level of processivity of the enzyme at 125 mM NaCl in comparison with that at 50 mM NaCl.

DISCUSSION

In this study, we have examined the behavior of AP endo on a multi-abasic site concatemeric substrate as well as on the 25-mer monomer from which the concatemer was constructed. The results indicate that enzymatic nicking of the monomer is independent of the sequence context, since the enzyme nicks the monomer from which the concatemer is formed in a fashion similar to the way it nicks a 49-mer substrate with a different sequence (*21*, *28*). Both steady state and single-turnover kinetic constants are similar to the previously published values (*21*, *28*). The fact that the catalytic efficiency remains at 5×10^7 M^{-1} s^{-1} for the 25-mer monomer implies that the interaction of the enzyme is diffusion-limited (*30*).

With the concatemer as the substrate, AP endo nicks on average seven or eight abasic sites, traversing a minimum of 175−200 base pairs, before it dissociates from the DNA. Unless AP endo cannot traverse an incised abasic site (or a nick in the DNA helix), which would tend to drive the enzyme in a single direction, we would expect the enzyme to move in a random fashion along the DNA, as is characteristic of diffusion-driven translocations (*31*). Hence, our method of estimating the distance traversed before the enzyme dissociates is likely to provide a minimum value. Our data do not address the issues of whether the enzyme binds initially at an abasic site and tracks from one abasic

site to the next or whether it binds to double-stranded regions of a DNA helix and scans for an abasic site. Our estimates of minimum distance traveled during a single association event agree with data published by Bennett et al. (*4*) and recalculated by us in which UDG at steady state excises eight or nine uracil residues before dissociating from its substrate, traveling at least 200−225 nucleotides in each cycle. This degree of processivity puts AP endo into the quasi processive category along with UDG (*4*), *Eco*RI (*32*), T4 endonuclease (*33*, *34*), and *Bam*HI (*35*), where quasi processivity refers to an enzyme that remains associated with DNA for up to several hundred base pairs in contrast to replicative DNA polymerases that remain associated with DNA for thousands of base pairs before they dissociate (*36*). These data do not determine whether processivity reflects the probability of enzyme dissociation from DNA between lesions, after catalysis, or both. Nevertheless, it is instructive that this methodology for measuring the ability to cleave multiple abasic sites on the same DNA molecule directly on a concatemeric substrate yields values similar to those provided by competition experiments for restriction and other endonucleases, where enzyme activity is measured on a multimeric substrate in the presence of large quantities of nonspecific DNA acting to compete for enzyme binding (*5*, *6*, *33*).

In these studies, we showed that nicking rates (k_{cat}) of both the monomer and the concatemer are similar. However, the nicking rate of the concatemer is probably a minimum estimate, because we could not quantitate nicks occurring randomly from an initial cleavage. Taking the average of eight cleavages per dissociation as an indicator of relative random cleavage and presuming that two cleavages occur to generate one 25-mer monomer, we found that the turnover number for the concatemer should be at least 12.5 s^{-1}. Although many processive enzymes demonstrate greatly enhanced rates on multimeric substrates with long stretches of nonspecific DNA (*31*, *37*), the enhancement is generally attributed to a reduced dissociation constant (k_{off}) for the binding of the enzyme to the multimeric substrate (*31*, *37*). We have shown that the kinetics of AP endo nicking a monomer double-stranded oligonucleotide can be described well by a Briggs−Haldane mechanism, where K_m is approximated as $(k_{off} + k_{cat})/k_{on}$. The parameters k_{on} and k_{off} refer to *substrate* binding and dissociation constants, respectively, that must be measured either under pre-steady state or single-turnover conditions in the absence of Mg^{2+}. They are entirely unrelated to dissociation of the *product* from the enzyme that occurs after the nicking reaction in the presence of Mg^{2+} and which would be described as k_{diss}. We were able to measure k_{on} and k_{off} directly from single-turnover measurements (*21*), where the nicking activity of the enzyme was limited to a single catalytic event. Since the k_{cat} value obtained from steady state measurements is 6 s^{-1}, which is 200 times k_{off}, a further diminution in k_{off} (0.03 s^{-1}) for the action of the enzyme on the concatemeric substrate would not make a major difference in either of the two steady state parameters (K_m and k_{cat}). Furthermore, since enzyme binding to the 25-mer monomer is already diffusion-limited, it is unlikely that binding to the concatemer will contribute to processivity. However, for processivity to occur, we presume that k_{diss}, the rate of dissociation of the enzyme from the substrate, has decreased. Since k_{diss} for AP endo is at least 100 times greater than k_{cat} with our kinetic simulations, a decrease in k_{diss} would not necessarily be accompanied by a change in k_{cat} on a multimeric substrate.

In our experiments, the nicking rate of abasic sites in the concatemer was separable from the scanning distance along the DNA. In fact, the degree of processivity was maximal at the lowest salt concentration that was employed (25 mM) and decreased linearly with increasing salt. In contrast, the rate of nicking was maximal at 125 mM NaCl, being 2 times faster at 125 mM than at 50 mM salt, which was in turn 2 times faster than that at 200 mM NaCl. This behavior is generally used as an indication of one-dimensional facilitated diffusion (*5*, *31*, *33*). Scanning or sliding is thought to operate by means of electrostatic interactions between the protein and the DNA molecule, where the attraction is between the acidic phosphodiester backbone and the basic amino acids of the protein. Since nicking activity is also salt sensitive, it is also dependent on electrostatic interactions between the protein and the DNA. However, the two sets of electrostatic interactions are differently affected by changing ionic strength. In fact, the processivity of AP endo is more sensitive to changes in salt concentration (loss of 30−40% of processivity when the NaCl concentration is increased from 25 to 100 or 125 mM) than is processivity of UDG, where the degree of processivity is not decreased by 30% until the NaCl concentration is raised to 300 mM. At 300 mM NaCl, AP endo, like *Eco*RI (*32*) and T4 endonuclease (*33*), is no longer functional, while UDG retains 25% of its initial uracil cleaving activity (*4*).

In the substrate used in these experiments, G/U pairs and, hence, adjacent abasic sites are 25 bp apart. Since one complete turn of a helix is ∼11 bp, adjacent abasic sites are too close for looping of the DNA to permit intersegment transfer (*31*). Furthermore, they are located on different faces of the helix. The presence of an abasic site in a DNA helix may or may not result in a kink, depending on the sequence context (*38*, *39*). Hence, we feel that AP endo probably follows the helical pitch of the DNA rather than simply scanning one face and is likely to recognize its substrate independently of any kink or distortion resulting from the sequence context in which the abasic site is situated.

We believe that the separability of the nicking rate from processivity is both biochemically and physiologically relevant. Salt concentration at or near isotonicity maximizes the ability of the enzyme to initiate repair of abasic sites. On the other hand, physiological salt concentrations are in the midrange of processivity behavior. Thus, this parameter can be modulated with precision. Since there is neither physical nor kinetic evidence for interaction of UDG and AP endo at this time, both AP endo and UDG need to be able to scan DNA independently. We believe that quasi processivity maximizes the independent scanning ability of these two repair enzymes with high turnover capacity without interfering with other repair proteins that may also need access to DNA lesions. Thus, quasi processivity plays an important role in the physiological maintenance of native DNA.

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