# Oligonucleotides with Bistranded Abasic Sites Interfere with Substrate Binding and Catalysis by Human Apurinic/Apyrimidinic Endonuclease†

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*Received July 19, 2001; Revised Manuscript Received September 4, 2001*

ABSTRACT: Apurinic/apyrimidinic endonuclease (AP endo) is a key enzyme in oxidative damage DNA repair. The enzyme, which repairs abasic sites, makes a single nick 5′ to the phosphodeoxyribose, leaving a free 3′-hydroxyl. We recently described single turnover kinetics for human recombinant AP endo acting on an oligonucleotide with a single abasic site. We hypothesized that the structural changes induced by the presence of a second abasic site might provide insight into how AP endo recognizes the first abasic site. Here we performed steady state and single turnover experiments using bistranded abasic site substrates, with the second site located on the complementary strand to the one being followed and either opposite to the first or displaced in the 5′ direction. All sites on the complementary strand were within half a helical turn of the first. The catalytic efficiency was reduced 80 to 96% and the $K_d$ for substrate binding and dissociation was elevated 40- to 125-fold. The smaller changes occurred when the second site was opposite the first site or displaced by four nucleotides. In addition, if the second abasic site was directly across the helix or displaced by 1 or 3 nucleotides from the first abasic site, cleavage of the first abasic site was subject to apparent *substrate* inhibition, which did not occur if the second abasic site was displaced by four nucleotides from the first. While a substrate containing a nick without a phosphodeoxyribose on the contralateral strand abasic site did not inhibit nicking of the first strand, a substrate with a *nicked abasic site* on the contralateral strand was an even stronger inhibitor of enzyme action than an oligonucleotide containing the corresponding abasic site on each strand. Consequently, the inhibitory effect of the second abasic site is probably the result of prior cleavage of the abasic site on the contralateral strand with resulting distortions to the DNA helix that interfere with enzyme binding and/or cleavage.

Abasic sites are lesions in DNA that arise from the hydrolysis of the N-glycosyl bond linking a nitrogenous base to the sugar−phosphate backbone (*1*−*5*). The frequency of abasic sites has been estimated as high as 10 000/cell/day (*6*). Abasic sites can be generated by spontaneous base loss (*4*) through DNA-damaging agents such as low level radiation (*6*, *7*), ionizing radiation, reactive oxygen species, and chemotherapeutic agents or through a group of DNA-repair enzymes called DNA glycosylases (*1*, *3*−*5*, *8*−*11*).

In eukaryotes, two forms of base excision repair (BER)[1] (reviewed in refs *2*, *5*, *12*, *13*) have been observed: the short-patch pathway characterized by the replacement of a single nucleotide and by its dependence primarily on DNA polymerase $\beta$ (pol-$\beta$) (*14*, *15*) and the long-patch pathway which involves the gap-filling of short stretches of DNA by pol-$\beta$ or polymerase $\delta$ or $\epsilon$ in conjunction with proliferating cell nuclear antigen (PCNA) and DNA endonuclease IV (flap endonuclease I or FEN 1) (*16*, *17*). The key enzyme common to both types of BER is apurinic/apyrimidinic endonuclease (AP endo) which recognizes and makes a single nick 5′ to an abasic site (*5*, *18*−*21*). Without AP endo, the 3′-hydroxyl required by the repair polymerase is lacking and synthesis cannot occur. AP endo has a second unrelated activity where it apparently behaves as a redox factor for transcription activating factors (*22*−*27*). The redox activity appears to be unrelated to the ability to cleave abasic sites (*27*). Because this enzyme could be pivotal for the entire ASR pathway (*5*, *25*), a thorough examination of the kinetics and mechanism of nicking is warranted. Steady-state (*18*, *20*, *28*−*32*) and single turnover (*20*, *28*, *30*) analyses of human AP endo have been performed. Steady-state assays (*18*, *20*, *28*−*32*) have determined $K_m$ and $k_{cat}$, while single turnover assays, followed by kinetic simulation, were used to obtain the substrate binding and dissociation constants $k_{on}$ and $k_{off}$, respectively (*20*, *28*, *31*). As is true of many other DNA repair enzymes including uracil DNA glycosylase (*33*), AP endo is processive when the substrate is a concatemeric substrate with multiple abasic sites (*28*).

Kinetic analysis including single turnover experiments is an extremely useful tool, which together with thermodynamic and structural data provides valuable information as to how an enzyme interacts with and acts upon its substrate. To date, we have applied single turnover kinetics only to substrates with a single abasic site (*20*, *28*, *31*). However, bistranded sites can be lethal, since in principle they could lead to double stranded breaks, and clustered DNA damage influences repair

† Supported by funds from the National Institutes of Health (CA 72702). A preliminary report of these findings has appeared (*54*).

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[1] Abbreviations: AP endo, apurinic/apyrimidinic endonuclease; BER, base excision repair; bp, base pair; dRP, phosphodeoxyribose; ds, double stranded; FEN I, DNA endonuclease IV; HDP, heat degradation product; NMR, nuclear magnetic resonance spectroscopy; PCNA, proliferating cell nuclear antigen; pol-$\beta$, DNA polymerase $\beta$; pTHF, 5′-phosphotetrahydrofurane.

10.1021/bi015587o CCC: $20.00 
Published on Web 10/16/2001

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A/B    A:  5′-32P-CT CTC CAT ACA UAC ATA TCC TCT-3′
       B:  3′-    GA GAG GTA TGT ATG  TAT AGG AGA-5′

A/C    A:  5′-32P-CT CTC CAT ACA UAC ATA TCC TCT-3′
       C:  3′-    GA GAG GTA TGT UTG TAT AGG AGA-5′

A/F    A:  5′-32P-CT CTC CAT ACA UAC ATA TCC TCT-3′
       F:  3′-    GA GAG GTA TGU ATG TAT AGG AGA-5′

A/F′   A:  5′-32P-CT CTC CAT ACA     UAC ATA TCC TCT-3′
       F′: 3′-    GA GAG GTA TGTp5′3′ATG TAT AGG AGA-5′

A/F′′  A:  5′-32P-CT CTC CAT ACA     UAC ATA TCC TCT-3′
       F′′:3′-    GA GAG GTA TGFp5′3′ATG TAT AGG AGA-5′

A/G    A:  5′-32P-CT CTC CAT ACA UAC ATA TCC TCT-3′
       G:  3′-    GA GAG GTA UGT ATG TAT AGG AGA-5′

A/V    A:  5′-32P-CT CTC CAT ACA UAC ATA TCC TCT-3′
       V:  3′-    GA GAG GTU TGT ATG TAT AGG AGA-5′
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FIGURE 1: Double-stranded 23-mer oligonucleotides used in this study. U indicates the presence of a uracil residue which was removed by UDG treatment to create an abasic site. In all cases, the abasic site being followed was the one at position 12 in strand A. A/B lacks a second abasic site, while in A/C the second abasic site is located across the helix from the first site. In A/F, the second site is located on strand F and displaced one nucleotide in the 5′ direction from the first (~45° around the helix). In A/G, the second site is located on strand G and displaced three nucleotides in the 5′ direction from the first (~135° around the helix), while in A/V the second site is located ~180° around the helix. In A/F′, strand F consists of two oligomers, one from 5′ nucleotide 1−12 and the other from nucleotide 13−23, where nucleotide 13 is a T, while in A/F″, strand F″ consists of nucleotide 1−12 and nucleotide 13−23, where nucleotide 13 is pTHF (F). See Figure 2 for three-dimensional representation of the location of the different U or F residues.

by prokaryotic enzymes and eukaryotic cell extracts (*36*). Furthermore, Chaudhry and Weinfeld demonstrated that they interfere with AP endo's nicking activity, although no mechanism was proposed (*34*, *35*). Interference is especially pronounced if the second abasic site is on the opposite strand (*21*, *34*, *35*) and 5′ to the first abasic site (*34*, *35*). In contrast, abasic sites located on the same strand as the initial one and 3′ to it or on the opposite strand and 3′ to the initial one interfere marginally with enzymatic activity (*35*). In this study, we performed a careful kinetic analysis of cleavage of bistranded abasic site-containing oligonucleotides, where the second abasic site was located within one-half a helical turn of the first site and on the opposite strand. The data describe how the type and degree of interference depend precisely on the location relative to the site being examined. Thus, they provide structural information on the enzyme/substrate interaction.

## MATERIALS AND METHODS

The substrates used for enzymatic reactions were either provided by Drs. Michael Weinfeld and Ahmad Chaudhry (*35*) or obtained from Genosys Biotechnologies (Woodlands, TX) (Figure 1). They consisted of 23-bp ds oligonucleotides containing either a single abasic site at position 12 of strand A (A/B) or two abasic sites, where the second abasic site was located on the strand complementary to A. One substrate contained a single abasic site with a nick on the contralateral (A/F′), while a similar substrate contained a single abasic site but a nick followed by the abasic site analogue, 5′-phosphotetrahydrofurane (pTHF) on the downstream complementary strand (A/F″). Since all experiments were

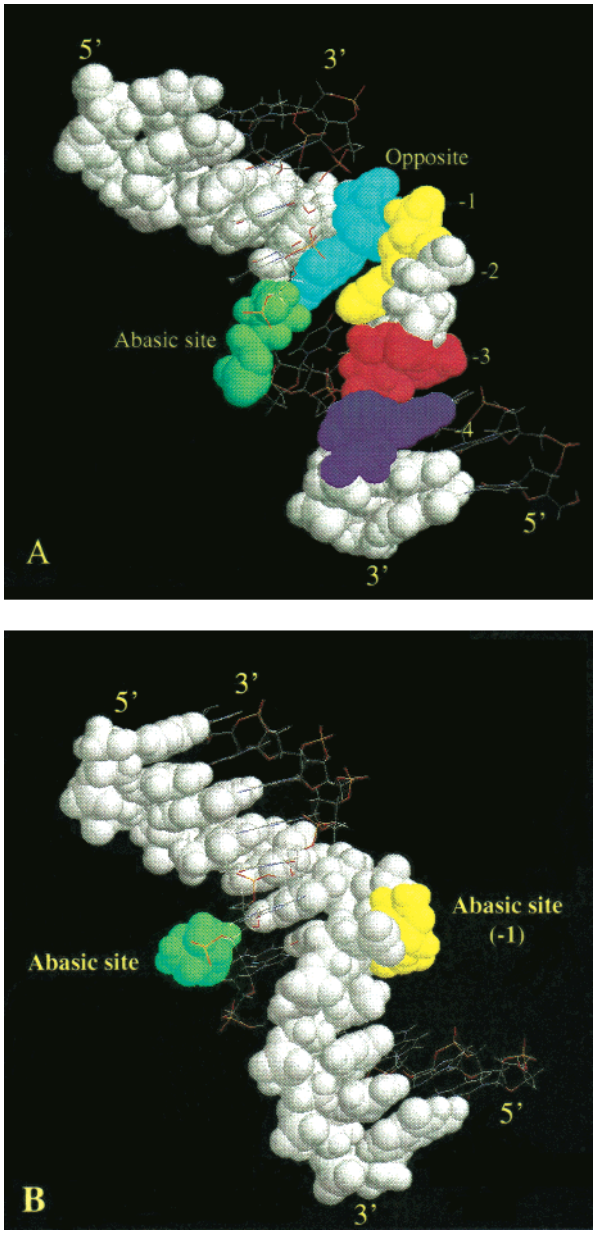


FIGURE 2: (A) Three-dimensional representation of a 12-mer double stranded oligonucleotide with a single abasic site obtained by NMR (*41*), illustrating the relative locations of potential abasic sites on the contralateral strand as described in Figure 1. Green, abasic site on strand A; cyan, nucleotide opposite the one on strand A; yellow, nucleotide located at −1 position (45°); red, nucleotide located at −3 position (135°); purple, nucleotide located at −4 (180°) position. (B) Three-dimensional representation of a 13-mer double stranded oligonucleotide with two abasic sites obtained by NMR (*52*). This figure illustrates a possible conformation for A/F, where the second abasic site is located at the −1 position.

performed with strand A labeled at the 5′ end, the only abasic site being followed was the one located on strand A. A/C was the duplex with the abasic sites directly opposite each other (0° displacement). The other substrates contained a second abasic site on the complementary strand displaced toward the 3′ end of that strand by 1 (45° displacement), 3 (135° displacement), and 4 (180° displacement) base pairs (A/F, A/G and A/V, respectively). To reinforce the importance of the steric relationship of the abasic sites on the complementary strand to the one on strand A, the former are referred to as being in the 5′ direction (relative to the

one on strand A). Enzyme was obtained by expression from pXC53 in *Escherichia coli* and purified as described (*20*).

Substrate containing one or more abasic sites was prepared as described previously (*20*, *28*, *31*) except that the complement was not added until the step where abasic sites were generated. Oligomer A was labeled at the 5′ end by means of T4 polynucleotide kinase (United States Biochemicals, Cleveland, OH) and [$\gamma^{32}$P] ATP (3,000 Ci/mmole, NEN Life Science Products, Boston, MA) in the buffer provided by the manufacturer. The reaction was allowed to proceed at 37 °C for 45 min after which time the enzyme was heat inactivated at 70 °C for 5 min. The labeled oligonucleotide was purified by phenol extraction and gel filtration chromatography (Sephadex G-50, fine; Pharmacia Biotech., Piscataway, NJ). The eluting buffer was 50 mM Hepes-NaOH, pH 7.5, 0.1 mM EDTA. To create the abasic site(s), [$^{32}$P]-labeled oligonucleotide A was mixed with the desired complement (B, C, F, F′, F″, G, or V) and incubated with uracil DNA glycosylase (1 U/100 pmol uracil residues) (Epicenter Technologies, Madison, WI) in a buffer consisting of 50 mM HEPES−NaOH, pH 7.5, 0.1 mM EDTA, and 0.1 mM freshly prepared $NaBH_4$ at 37 °C for 30 min. The purpose of $NaBH_4$ was to stabilize the abasic site as deoxyribitol, which was not affected by the following heat treatment. The reaction was terminated by heating at 70 °C for 5 min and slow cooled to room temperature. Since UDG does not perform a lyase reaction, the presence of $NaBH_4$ does not interfere with its activity (*37*, *38*). Control experiments with substrates labeled on both strands indicated that the presence of uracil residues on contralateral strands did not interfere with the ability of UDG to remove uracil (data not shown).

*Steady-State Assays*. Steady-state time dependence assays were carried out as described (*20*, *28*, *31*). Except as noted, enzyme was incubated with substrate at room temperature for 5−120 s in a mixture containing 50 mM HEPES−NaOH, pH 7.5, 90 mM NaCl, 5 mM $MgCl_2$, 0.5 or 1 nM AP endo, and 260 nM ds DNA substrate in a final volume of 5 $\mu$L. To ensure complete annealing, experiments using substrates A/F′ and A/F″ were performed at 18 °C. The reaction was terminated by the addition of EDTA to a final concentration of 83 mM. Substrate and product were resolved by denaturing polyacrylamide gel electrophoresis (15% gel) in the presence of 8 M urea and quantitated by phosphorImager analysis by means of a Storm 840 system (Molecular Dynamics, Sunnyvale, CA). Steady-state concentration dependence assays were performed in the same buffer and volume at room temperature for 20 s with substrate concentrations between 0.016 and 1.33 $\mu$M and a final enzyme concentration of 0.5 or 1.0 nM.

*Single-Turnover Assays*. Single turnover assays were performed as described by Strauss et al. (*20*) except that HDP used to prevent a second round of enzyme catalysis was prepared from an 11-mer with the sequence 5′-ACG AAU TAA GC-3′; 3′-TGC TTG ATT CG-5′.

Enzyme (4 nM) and substrate (4 nM) were allowed to bind in the presence of 4 mM EDTA for 5−40 s intervals at which time 10 mM $MgCl_2$ and trap (2 mg/mL heparin and 23 $\mu$M HDP, final concentration) were added to allow cleavage of substrate that was already bound to the enzyme and prevent recycling of free enzyme. The final volume was 5 $\mu$L. After a 20 s incubation with $Mg^{2+}$/trap, the reaction was terminated by adding 1 $\mu$L of 0.5 M EDTA to a final concentration of 83 mM.

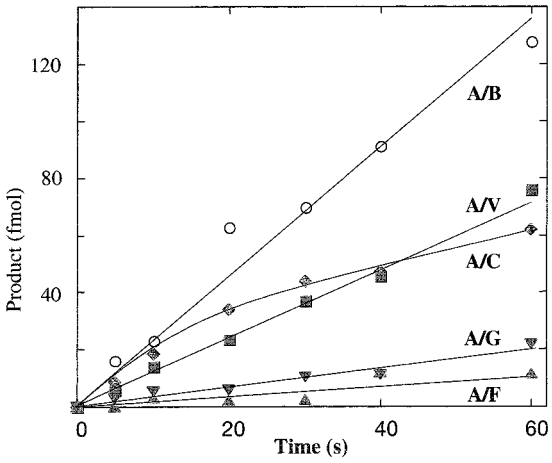


FIGURE 3: Time dependence of cleavage of bistranded abasic site-containing 23-mers by AP endo. In all cases, the abasic site being followed was located on strand A. Substrate was present at 260 nM, while enzyme was present at 0.5 or 1.0 nM. All values were normalized to 0.5 nM enzyme. Time was varied between 0 and 60 s. These data are the average of two experiments for A/G and A/F and four experiments for A/B, A/C, and A/V.

*Data Analysis*. All experiments were repeated a minimum of twice and in some cases 5 or 6 times. For steady-state assays, data were fitted to appropriate equations with KaleidaGraph. The Michaelis constant $K_m$ and turnover number $k_{cat}$ were calculated from the intercepts of the Lineweaver−Burk plots obtained at or below substrate concentrations of 400 nM (See text). Since most of the bistranded abasic site-containing substrates used in this study were subject to substrate inhibition, the $K_m$, $V_{max}$, and $k_{cat}$ values are reported as $K_{m,app}$, $V_{max,app}$, and $k_{cat,app}$. Association and dissociation rate constants ($k_{on}$ and $k_{off}$, respectively) were determined from simulations with HopKINSIM (*39*). Graphic visualization of the possible relationship of the second abasic site to the first was performed using Rasmol (*40*) or Swiss PDB Viewer from coordinates for oligonucleotides containing a single abasic site (*41*) and for those containing bistranded abasic sites kindly provided in advance of publication by Dr. Carlos de los Santos.

## RESULTS

*Steady-State Analysis*. To examine the effects of a second abasic site on the enzymatic activity of AP endo, we compared the time dependence of nicking at 260 nM of different substrates either lacking a second abasic site (A/B, see Figures 1 and 2) with that of substrate with a second abasic site located at strategic positions relative to the first (A/C, A/F, A/G, and A/V). In each case, the label was located on the 5′ end of strand A, which contained the abasic site at position 12, and, when present, the second abasic site was on the strand opposite the radiolabeled strand. Consequently, the visualized product was always an 11-bp oligomer with a hydroxyl group located at the 3′ end. As described earlier by Chaudhry and Weinfeld (*34*, *35*), the presence of a second abasic site was deleterious to nicking of the first, since the rate of nicking of all the bistranded abasic site-containing substrates was reduced from that of an oligonucleotide containing a single abasic site (Figure 3).

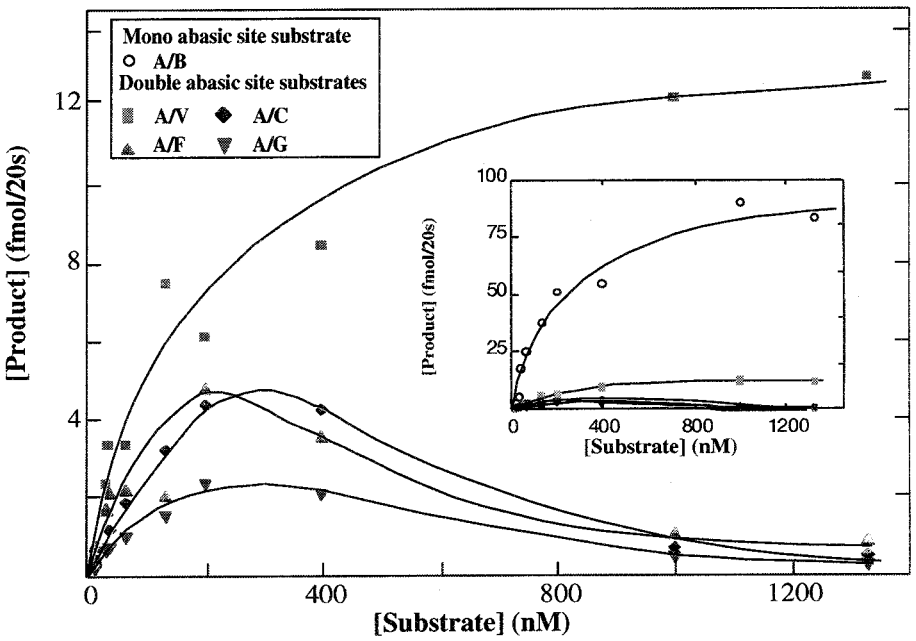


FIGURE 4: Concentration dependence of cleavage of bistranded abasic site-containing 23-mers by AP endo over a 20 s interval. Substrate concentration was varied between 16 and 1500 nM, while enzyme was present at either 0.1 or 0.2 nM. All values were normalized to rates for 0.1 nM enzyme. Each line represents the average of 3−5 experiments.

We then investigated the concentration dependence of nicking the five substrates (Figure 4). In all cases, the presence of a second abasic site on the complementary strand inhibited nicking of the first abasic site located on strand A. If the presence of the second abasic site were solely the result of doubling the number of abasic sites on which the enzyme could act, one would expect that the apparent nicking rate to change commensurate with the increased substrate concentration and the halved specific activity. Also, the position of the second abasic site should not be relevant. However, the kinetic picture indicated that the second abasic site disturbed the cleavage of the first site in a complex manner, depending on the location of the second site (Figures 3 and 4). The maximum velocity for substrate A/V was reached at 1 $\mu$M. The maximum velocity for A/C, A/F, and A/G substrates was reached at 200−400 nM substrate and declined at higher concentrations. A second abasic site located directly across the helix (A/C) or displaced by four nucleotides (A/V) reduced nicking of the first abasic site at 200 nM oligonucleotide by 91 and 88%, respectively. With a second abasic site across the helix from the first, the ratio of product at 1 $\mu$M substrate to that at 200 nM was 0.15, while with substrates A/F and A/G, the ratios were 0.21 and 0.17, respectively. Thus, substrates where there was no second abasic site or where the abasic site was displaced by four nucleotides across from the first were not subject to inhibition at high substrate concentrations in contrast to substrates where the second abasic site was opposite to or offset from the first in the 5′ direction by one or three nucleotides. This pattern will become even sharper in the data presented below (Table 2).

Because the initial rate of cleavage was reduced above 200−400 nM substrate, $V_{max}$ and $k_{cat}$ could only be approximated by a Lineweaver−Burk plot obtained at low substrate concentrations. However, the slope of the concentration vs velocity curve at low concentrations is a true reflection of enzymatic efficiency, $k_{cat}/K_m$ (*40*). Furthermore,

Table 1: Steady State Parameters $K_{m,app}$, $k_{cat,app}$, and $k_{cat,app}/K_{m,app}$ and the $K_{d,app}$ for the Different Bistranded Abasic Sites

| substrate | $k_{cat,app}$ ($s^{-1}$) | $K_{m,app}$ (nM) | $k_{cat,app}/K_{m,app}$ ($M^{-1}\,s^{-1}$) | $K_{d,app}$ (nM) |
|---|---|---|---|---|
| A/B | $9 \pm 0.8$ | $170 \pm 16$ | $5 \times 10^7$ | 0.8 |
| A/C | $0.9 \pm 0.04$ | $240 \pm 19$ | $3 \times 10^6$ | 72 |
| A/F | $0.8 \pm 0.3$ | $180 \pm 25$ | $4 \times 10^6$ | 220 |
| A/G | $0.3 \pm 0.02$ | $110 \pm 12$ | $2 \times 10^6$ | 340 |
| A/V | $1.1 \pm 0.4$ | $125 \pm 5$ | $1 \times 10^7$ | 38 |

Table 2: Substrate Binding and Dissociation Constants and Corrected $K_d$ Values Obtained by Kinetic Simulation

| substrate | $K_d$ (nM) (corrected) | $k_{on}$ ($M^{-1}\,s^{-1}$) | $k_{off}$ ($s^{-1}$) |
|---|---|---|---|
| A/B | 0.8 | $5 \times 10^7$ | 0.04 |
| A/C | 58 | $3 \times 10^6$ | 0.2 |
| A/F | 100 | $5 \times 10^6$ | 0.5 |
| A/G | 60 | $2 \times 10^7$ | 1.0 |
| A/V | 32 | $8 \times 10^6$ | 0.3 |

with an enzyme that follows Briggs-Haldane kinetics as does AP endo (*20*), enzymatic efficiency usually reflects $k_{on}$ for binding of substrate to enzyme. For comparison purposes with a single abasic site-containing substrate (A/B), we obtained $k_{cat,app}$ values, calculated as $V_{max,app}/[E]$ and $K_m$ values for the bistranded abasic site substrates. The presence of a second abasic site inhibited nicking by 90% (A/C), 92% (A/F), 97% (A/G), and 88% (A/V) (See Table 1.). Again the decrease in velocity was far greater than that which could be attributed to a doubling of substrate molecules. Furthermore, location of the second abasic site displaced by 180° was less inhibitory than if the second site were directly across or displaced by 45° or 135° around the helix (one or three residues). Finally, the shapes of the substrate dependence curves were unique to each oligomer and depended on the location of the second abasic site relative to the first (Figure 4). Provided that the substrate concentration did not exceed

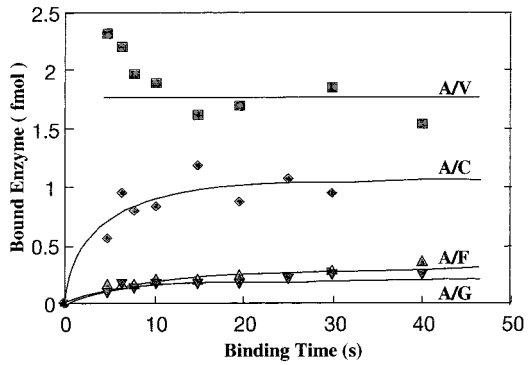


FIGURE 5: Single turnover kinetics for bistranded abasic-site containing 23-mers. Substrate and enzyme were each present at 4 nM. These data are the average of two or three experiments for each substrate.

400 nM, the $K_{m,app}$ values varied between an increase of 40% over that of A/B when A/C was used as substrate to a decrease of 35% on the part of A/G, while the $K_{m,app}$ for A/F and for A/V remained largely unchanged. The catalytic efficiencies of all the bistranded abasic site-containing substrates obtained directly from the initial slope of the concentration vs velocity plot or from the calculated $k_{cat,app}/K_{m,app}$ obtained from the Lineweaver−Burk plot were reduced from that of the control substrate by approximately 1 order of magnitude, so long as the substrate concentration did not exceed 400 nM.

*Single Turnover Analysis*. To explore the basis for the inhibitory effects of bistranded abasic sites on nicking by AP endo, we examined binding and dissociation of substrate under single turnover conditions (Figure 5). In these experiments, 4 nM substrate was allowed to bind to 4 nM enzyme in the presence of 4 mM EDTA, which permitted binding but prevented cleavage. The enzyme was then released to perform the catalytic step by the addition of 10 mM $Mg^{2+}$ in the presence of trap (*20*, *28*, *31*). The trap prevented recycling so that only a single enzymatic turnover was observed. The equilibrium level of [E·S] for a substrate with a single abasic site (A/B) was 0.29 nM with a corresponding $K_d$ of 0.8 nM, measured at 0.4 nM enzyme and 4 nM substrate, conditions chosen because of the efficiency of binding (*20*). Under the conditions used in this study, 4 nM substrate and 4 nM enzyme, the equilibrium level of [E·S] would have been 2.6 nM, as determined by kinetic simulation. In all cases the presence of a second abasic site markedly reduced the measured equilibrium level of [E·S] and the degree of reduction depended on the location of the second abasic site. Specifically, the observed equilibrium level of [E·S] was 0.2 nM for A/C, 0.07 nM for A/F, 0.046 nM for A/G, and 0.35 nM for A/V. (Note that the data in Figure 5 are presented as fmol/reaction.) If the second abasic site was across the helix or displaced by 180° from the first, the increase in $K_{d,app}$ (Table 1) was least severe (47- or 90-fold) than if the second abasic site was displaced by 45° (275-fold) or 135° (425-fold) bases. However, the measured values are apparent values, since the rate of dissociation of substrate from the enzyme−substrate complex was on the same order of magnitude as the forward rate constant, $k_{cat}$.[2] The corrected values are discussed below.

Besides altering the equilibrium level of [E·S], the presence of a second abasic site altered the rate at which binding equilibrium was approached. Since the shortest time point possible with our current methodology was 5 s, we could only determine a $t_{1/2}$ for substrates A/C and A/F, where the $t_{1/2}$ was 5 and 11 s, respectively. Nevertheless, kinetic simulation coupled with the equilibrium level of the [E·S] allowed us to calculate the minimum association ($k_{on}$) and maximum dissociation ($k_{off}$) rate constants shown in Table 2. Comparison of the calculated values agreed well with the enzymatic efficiencies obtained from concentration vs velocity curves at low concentrations. Bistranded abasic site containing substrates increased the dissociation constant by at least 5-fold, while the association constant was decreased up to 17-fold (A/C). These changes are reflected in the $K_d$ values shown in Table 1. Comparison of these values with the observed catalytic constant ($k_{cat,app}$) allowed us to correct [ES] values and to obtain the corrected $K_d$ (Table 2). The bistranded abasic site-containing substrate with the second site across from the first (A/C) had the greatest effect on substrate binding ($k_{on}$), while those displaced by 45° or 135° (A/G and A/F) had the greatest effect on substrate dissociation ($k_{off}$). Furthermore, since enzymatic efficiency is a measure of substrate binding at substrate concentrations well below the $K_m$ value (*42*), it is noteworthy that the only substrate for which the $k_{on}$ and $k_{cat,app}/K_{m,app}$ disagreed was A/G, which caused the greatest inhibition of $k_{cat,app}$ and the largest increase in measured $K_d$.

Since AP endo requires a double-stranded substrate, we wondered whether these results could be explained by the presence of a simple nick generated on the contralateral strand before the enzyme interacts to cleave the abasic site on strand A. We also wondered whether substrate containing a nick followed by an abasic site would still generate results similar to those described above. Since a tetrahydrofuran (THF) residue in an oligonucleotide is an excellent substrate for AP endo (*21*, P. Strauss, unpublished data), we constructed the complementary strand to mimic a cleaved abasic site. The presence of a single nick without an abasic site (A/F′) altered cleavage of substrate containing a single abasic site to a small degree (Figure 6). The presence of a nick followed by pTHF (A/F″) resulted in even greater inhibition than the corresponding bistranded oligonucleotide (A/F). Thus, the effect of the second abasic site located on the contralateral strand was probably due to nicking of the contralateral abasic site in advance of binding to strand A. This observation may also explain the apparent substrate inhibition observed at high concentrations of A/C, A/F, and A/G. That is, accumulation of a nicked abasic site across from the observed abasic site inhibited cleavage of the test site.

[2] In the Briggs Haldane scheme,

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} EP \xrightarrow{k_{+3}} E + P$$

$K_d$ which is $k_{-1}/k_{+1}$, is determined by allowing enzyme to bind to substrate to form ES in the absence of $Mg^{2+}$. The amount of ES is measured by releasing the reaction with $Mg^{2+}$. We prevent the enzyme from going through more than one cycle by adding a trap (*20*, *42*). Once $Mg^{2+}$ is added, the forward rate is a function of $k_{+2}$ which in this case is $k_{cat}$. However, if $k_{-1}$ and $k_{+2}$ are the same order of magnitude, [ES] will be underestimated, because substantial amounts of ES will revert to E+S. Consequently, the $K_d$ will be overestimated. However, the extent of overestimation can be determined by kinetic modeling.

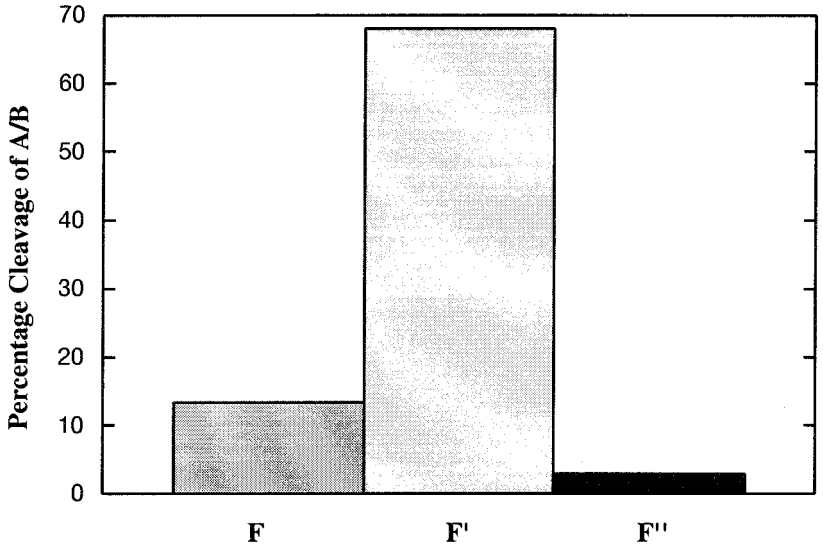


FIGURE 6: Effect of a nick (substrate A/F″) or a nick followed by pTHF (substrates A/F″) on the downstream strand of the contralateral strand on cleavage by AP endonuclease. Substrate was present at 200 nM, while enzyme was present at 0.2 nM (A/B) or 2.0 nM (A/F′, A/F″). Turnover for A/B under these conditions was 1.1 $s^{-1}$. The data are the average of two experiments, carried out at 18 °C, which is below the $T_m$ for the individual oligonucleotides.

## DISCUSSION

We are interested in identifying the steric requirements for human AP endo recognition and cleavage of an abasic site. Although the crystal structures of the protein with an 11-mer oligonucleotide substrate (*43*) and without substrate (*44*, *45*) are available, these do not reflect dynamic conformations that either the enzyme or the substrate is likely to undergo during catalysis. We focused on the five nucleotides upstream of the abasic site which are required for maximal activity (*21*, *34*, *35*, *46*, *47*), since Chaudhry and Weinfeld (*34*, *35*) had demonstrated that a second abasic site at these positions had more profound effects than in other positions. In these studies, we used single turnover and steady-state kinetic measurements to assess nicking when a second abasic site was located in selected positions on the strand complementary to the one where the reporter abasic site was located. All the bistranded substrates reduced catalysis and increased the $K_d$ beyond that which would have been expected merely by the presence of additional abasic sites. The precise effect depended on the location of the second abasic site relative to the first.

NMR studies on an 11-mer containing a single abasic site opposite either an A or a C residue indicate that the conformation of an abasic site-containing oligomer depends both on the base opposite the abasic site and the sequence context in which the abasic site is located (*39*, *48*−*51*). The presence of a water molecule stabilizes a structure closer to B-form DNA when the base opposite the first is an A in contrast to a C. However, the abasic site can also assume a flipped out configuration from the helix which is associated with a bend in the helix. More recent NMR studies examined the structure of several bistranded abasic site-containing 13-mers. Of particular relevance is the structure equivalent to A/F (Figure 2B). In this case, the two abasic sites are squeezed out of the helix and their complementary bases pair with each other while the helical axis remains unchanged (*49*). Thus, while AP endo may have little or no sequence selectivity and may impose a transition state structure on any abasic site-containing oligonucleotide to which it binds, dynamic distortions caused by the second abasic site may interfere with the ability of the enzyme to bind the first one.

We wondered whether the effects of the second abasic site on cleavage of the first might be the result of cleavage of the second *prior* to the enzyme recognizing the abasic site on strand A. In that case, the substrate would be a double stranded oligonucleotide containing an abasic site with a *nicked abasic site* on the contralateral strand. Since AP endo requires a double stranded substrate, it might also be possible that the mere presence of a nick on the complementary strand would suffice to produce the inhibitory kinetics we observed. In fact, a nicked contralateral strand without an abasic site was a fairly good substrate, although not quite as good as a substrate without the nick. However, a nicked contralateral strand with an abasic site analogue on the downstream strand was an even poorer substrate than the corresponding bistranded abasic site.

There are several possibilities that might explain these results. One involves physical blockage by a first enzyme molecule of cleavage at a second site by a second enzyme molecule; the other involves changes in conformation of the DNA itself, either because of the presence of two bistranded abasic sites or of one abasic site on one strand and a cleaved abasic site on the contralateral strand. In regard to blockage of one enzyme by another, the first enzyme molecule to cleave the first abasic site could bind tightly to its product and block the second enzyme from binding and cleaving an abasic site on the contralateral strand or the first enzyme molecule to cleave the first abasic site might bind tightly to its product, while the second enzyme might bind but be unable to cleave the abasic site on the contralateral strand. Both cocrystal (*43*) and molecular footprinting studies (*46*, *47*) make it unlikely that more than one enzyme molecule can bind simultaneously in the region of an abasic site (within half a turn of the DNA helix). Furthermore, the studies showing that the cleaved abasic site on the contralateral strand is an even more deleterious substrate than one with a

second abasic site imply that the enzyme would have to bind *preferentially* to the dRP residue in order to block cleavage at the reporter abasic site. However, stability of the enzyme−product complex with standard substrate under kinetic conditions is associated with a fairly rapid $k_{off}$ of 0.04 $s^{-1}$ and a rapid turnover of 10 $s^{-1}$ (*20*). What makes the argument of interference or stabilization unlikely as a primary mechanism is that the decrease in turnover number for the bistranded substrates is accompanied by an *increased* $K_d$ of 40−125-fold and an *increased* $k_{off}$ of 5−25-fold.

The second possibility involves conformation of the DNA substrate itself. In fact, once steady-state conditions are established for any given bistranded abasic site-containing oligonucleotide, there are *two* potential substrates: (i) both abasic sites are intact with binding to and cleavage of the first abasic site being random; (ii) one abasic site is intact and one abasic site is already cleaved. Changes in the conformation of the bistranded abasic site-containing oligonucleotide might diminish binding and cleavage at *both* sites because of the presence of the two abasic sites and their location relative to each other (*52*), as shown in Figure 2B and described above Alternatively, changes in the conformation of the bistranded abasic site-containing oligonucleotide might diminish binding and cleavage of the *second* abasic site because of alterations due to a cleaved abasic site on the contralateral strand. Since the presence of a cleaved abasic site (dRP residue) on the contralateral strand was an even poorer substrate than the second abasic site itself, we feel that the distortions arising from the presence of two abasic sites might explain part of our observations but certainly not all of them. Rather, we propose that the selective interference of a cleaved abasic site on the contralateral strand is best explained by relaxation of the initial distortion caused by the first abasic site to be cleaved, freeing the abasic site, *which cannot base pair and may fail to tuck into the helix*. Thus, the conformation required by the enzyme to bind substrate or the transition state structure might be difficult to achieve. Furthermore, since the cocrystal (*43*) demonstrates that bases within half a helical turn of the abasic site and on the opposite strand are associated with specific AP endo residues $Met^{271}$, $Lys^{78}$, $Lys^{98}$, $Ala^{74}$, and $Thr^{97}$, the dRP residue *in particular positions on the contralateral strand* may inhibit enzyme binding and cleavage. Finally, both our domain mapping studies (*53*) and our mutagenesis studies (*31*) suggest that enzyme and substrate each undergo conformational change during the process of nicking the abasic site.

These results confirm and extend the observations suggesting that conformation of DNA within 5 Å of the abasic site, that is, the half a helical turn immediately upstream from the abasic site, is critical to the enzymatic activity of human recombinant AP endo.

## ACKNOWLEDGMENT

The authors are grateful to Dr. William Beard for helpful discussions during the course of this work, to Drs. Michael Weinfeld and Ahmad Chaudhry for the gift of oligonucleotides used to make bistranded abasic site-containing substrates, and to Dr. Carlos de los Santos for providing the molecular coordinates for bistranded abasic sites obtained by NMR spectroscopy in advance of publication.

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BI015587O