

# Substrate Binding by Human Apurinic/Apyrimidinic Endonuclease Indicates a Briggs-Haldane Mechanism\*

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**Apurinic/aprimidinic endonuclease (AP endo) makes a single nick 5' to a DNA abasic site. We have characterized this reaction by steady-state and transient-state kinetics with purified human AP endo, which had been expressed in *Escherichia coli*. The substrate was a 49-base pair oligonucleotide with an abasic site at position 21. This substrate was generated by treating a 49-mer duplex oligonucleotide with a single G/U located at position 21 with uracil-DNA glycosylase. The enzymatic products of the AP endo nicking reaction were a 20-mer with a hydroxyl group at the 3'-terminus and a 28-mer with a phosphodeoxyribose at the 5'-terminus. To obtain maximal enzymatic activity, it was necessary to stabilize the abasic site during treatment with uracil-DNA glycosylase with a reducing agent. Otherwise, a 20-mer with phosphoribose at the 3'-terminus resulted from  $\beta$ -elimination. In agreement with others,  $K_m$  and  $k_{cat}$  were 100 nM and  $10\text{ s}^{-1}$ , respectively. Heat treatment of the abasic site-containing 49-mer without enzyme also resulted in conversion to the  $\beta$ -elimination product. The resultant heat degradation product was an efficient inhibitor of AP endo with a  $K_i$  of 30 nM. The enzyme required divalent cation ( $\text{Mg}^{2+}$ ) for activity, but bound substrate DNA in the absence of  $\text{Mg}^{2+}$ . Electrophoretic mobility shift assays indicated that AP endo bound tightly to DNA containing an abasic site and formed a 1:1 complex at low enzyme concentrations. The association and dissociation rate constants for substrate binding to AP endo were determined by using a challenge assay to follow AP endo-substrate complex formation. Heat degradation product together with heparin served as an effective trap for free enzyme. The results are consistent with a Briggs-Haldane mechanism where  $k_{on}$  and  $k_{off}$  are  $5 \times 10^7\text{ M}^{-1}\text{ s}^{-1}$  and  $0.04\text{ s}^{-1}$ , respectively ( $K_d = 0.8\text{ nM}$ ),  $k_{cat}$  is  $10\text{ s}^{-1}$ , and product release is very rapid (*i.e.*  $k_{off,product} \gg 10\text{ s}^{-1}$ ). This scheme is in excellent agreement with the measured steady-state kinetic parameters.**

Human apurinic/aprimidinic endonuclease (AP endo)<sup>1</sup> (EC 3.1.25.2) is a bifunctional enzyme with the ability both to initiate repair of abasic sites in damaged DNA and to act as a

redox protein in restoring promoter binding to oxidation-damaged Jun-Fos or Jun-Jun dimers (1, 2). The gene for human AP endo has been identified (3–6), and several recombinant AP endos have been expressed (4–6). The open reading frame encodes a protein of 319 amino acids and a molecular mass of 35.5 kDa. In this report, we focus on the endonuclease function that is found in the carboxyl-terminal 250 residues (2, 7). AP endo makes a single cleavage in the phosphodiester backbone on the 5'-side of the abasic site created by any of several DNA glycosylases and is, therefore, classified as a class II endonuclease (Fig. 1) (8–10). Subsequently, in mammalian base excision repair, the phosphodeoxyribose remaining attached to the 5'-end of the downstream DNA strand is removed by the amino-terminal 8-kDa domain of DNA polymerase  $\beta$  (11) in order for this polymerase to insert a nucleotide opposite the exposed base on the complementary strand (12, 13).

The endonuclease activity of AP endo has been well characterized for its preference for abasic site-containing DNA and by steady-state kinetic analysis in the presence of divalent cation. In order to understand the kinetic basis for enzyme specificity and efficiency, each step in the reaction pathway needs to be characterized thermodynamically and kinetically. This is a prerequisite to rational drug design in developing efficient enzyme inhibitors to down-regulate DNA repair. In this paper, we examine substrate binding in detail to derive association and dissociation rate and equilibrium constants. These studies were facilitated by the observation that the spontaneous heat degradation product (HDP) of the oligonucleotide containing an abasic site is an effective inhibitor of human AP endo. The HDP was employed in a challenge assay to measure enzyme-DNA complex formation. Product complexes could not be formed during catalytic assays, indicating that product release was much faster than the nicking reaction. These results suggest a simple Briggs-Haldane mechanism for AP endo.

## EXPERIMENTAL PROCEDURES

**Enzymatic Assays**—The substrate for all enzyme assays, obtained either from Operon Technologies, Inc. (Alameda, CA) or from Genosys (Woodlands, TX), was a 49-base pair (bp) oligomer containing a single G/U base pair at position 21.

5'-AGC TAC CAT GCC TGC ACG AAU TAA GCA ATT CGT AAT CAT GGT CAT AGC T-3'  
3'-TCG ATG GTA CCG ACG TGC TTG ATT CGT TAA GCA TTA GTA CCT GTA TCG A-5'

## SEQUENCE 1

The single-stranded oligomer was labeled at the 5'-end of the U-containing strand by means of T4 polynucleotide kinase (New England Biolabs) and [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Corp.) in the buffer supplied by the manufacturer. End-labeling was performed at 37 °C for 45 min. In some cases, the 49-mer was labeled at the 3'-end by means of terminal deoxynucleotidyl transferase (Promega Corp.) and [ $\alpha$ -<sup>32</sup>P]ddATP (Amersham Corp.). The abasic site was created by treating the double-stranded oligomer with uracil-DNA glycosylase (Epicentre Technologies) at 1 unit of enzyme/100 pmol of uracil residues, for 15–30 min at 37 °C. DNA substrate was quantified spectrophotometrically. The buffer consisted of 50 mM HEPES-NaOH, pH 7.5, 0.1 mM EDTA, and 0.1 mM

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<sup>1</sup> The abbreviations used are: AP endo, apurinic/aprimidinic endonuclease; HDP, heat degradation product; bp, base pair(s); ES, enzyme-substrate.

freshly prepared  $\text{NaBH}_4$ , as indicated. The reaction was terminated by heating at 70 °C for 5 min. The mixture was allowed to cool slowly to room temperature.

Steady-state enzyme assays were carried out at 25 °C for 5–120 s in 50 mM HEPES-NaOH, pH 7.5, 25 mM NaCl, 5 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, 0.02–20 nM AP endo, and 150 nM DNA in a volume of 5  $\mu\text{l}$ . Assays were terminated either by the addition of urea-containing gel loading dyes or with EDTA to a final concentration of 70–85 mM. Products were resolved by gel electrophoresis using 8 M urea, 15% polyacrylamide gels (14), followed by autoradiography, and quantified with a Molecular Dynamics PhosphorImager (Sunnyvale, CA). In some cases, products were restabilized before electrophoresis by treatment with 0.1 M  $\text{NaBH}_4$  for 30 min at 0 °C, followed by ethanol precipitation in the presence of 0.2  $\mu\text{g/ml}$  tRNA. Enzyme activity was linear for up to 60 s in the range of 0.02–10 nM AP endo.

**Challenge Assay**—AP endo could be limited to a single turnover by the addition of an enzyme trapping agent. Enzyme was preincubated with substrate, but without  $\text{Mg}^{2+}$ , to form enzyme-substrate complex in the presence of 4 mM EDTA. Productive complex was then assayed by the addition of excess  $\text{Mg}^{2+}$  and trap (*i.e.* heparin and HDP). Heparin and HDP bind to free enzyme, as well as enzyme that dissociates from the substrate during the course of the reaction, making the dissociation reaction irreversible.

Transient-state assays to determine the association and dissociation rate constants were carried out under single-turnover conditions. To determine the association and dissociation rate constants,  $k_{\text{on}}$  and  $k_{\text{off}}$ , respectively, enzyme and substrate were incubated for various periods up to 30 s under pseudo-first order conditions (*i.e.* substrate/enzyme = 10). At the end of each period, the amount of enzyme-DNA complex was measured by initiating enzymatic activity with the addition of  $\text{Mg}^{2+}$  (10 mM final concentration) and trap and incubating for an additional 10 s. The final volume was 5  $\mu\text{l}$ . The reaction was terminated by the addition of EDTA to a final concentration of 87 mM. Further details are outlined in the figure legend.

**Electrophoretic Mobility Shift Assay**—Substrate DNA was prepared as described above, except that  $\text{NaBH}_4$  was omitted during generation of the abasic site. To prevent the formation of HDP, the reaction was not heated to terminate the uracil-DNA glycosylase step. Substrate DNA was incubated with AP endo at different concentrations for 45 min at 25 °C in the presence of 0.5% polyvinyl alcohol, 10% glycerol, 100  $\mu\text{g/ml}$  bovine serum albumin, 8 mM NaCl, 50 mM HEPES-NaOH, pH 7.5, 0.1 mM EDTA, and 5 mM  $\text{MgCl}_2$ . The inclusion of  $\text{MgCl}_2$  was not necessary for binding. The incubation mixture was loaded onto a 7% nondenaturing polyacrylamide gel, and components were resolved by electrophoresis at 90 V (7 V/cm) for 6 h. The distribution of oligodeoxynucleotide bound and unbound forms was quantified by PhosphorImager analysis.

**Data Analysis**—Data were fitted to appropriate equations by nonlinear least squares methods. The association rate constant and steady-state time courses were simulated with HopKINSIM (15), a Macintosh version of the kinetic simulation program KINSIM (16).

The association and dissociation rate constants were determined from the binding curves determined under pseudo-first order conditions. Assuming a simple one-step binding model ( $E + S \rightleftharpoons ES$ ), the data should conform to a simple exponential where  $k_{\text{obs}} = k_{\text{on}}[S] + k_{\text{off}}$ . The association and dissociation rate constants were determined from a secondary plot of  $k_{\text{obs}}$  versus  $[S]$ .

**Expression Plasmid Construction**—The HAP1h cDNA (3) was amplified by polymerase chain reaction from HeLa cell DNA using the following primers: 5'-AAAAATGCATATGCCGAAACGTGG-3' (BAP.NsiI) and 5'-CTCTTAATTAATCACAGAGCTAGGTATAGGGTAA-3' (BAP.PacI).

The BAP.NsiI primer added an NsiI restriction site (underlined) and created an NdeI restriction site (italicized) upstream of the coding sequence, which is shown in bold. The BAP.PacI primer added a PacI restriction site (underlined) downstream of the coding sequence. The resulting polymerase chain reaction product was digested with NsiI and PacI and inserted into plasmid vector pXC20PacI, which had been digested with the same two enzymes. Vector pXC20PacI is a derivative of the phage  $\lambda\text{P}_L$  promoter vector pXC36 (17) and will be described elsewhere. The resulting plasmid was named pXC49, and the nucleotide sequence of the insert was confirmed by DNA sequencing. A restriction fragment containing the entire HAP1h coding sequence plus a short segment of the vector 3' to the coding sequence was prepared by complete digestion of pXC49 with BamHI followed by partial digestion with NdeI. The fragment was inserted into the phage T7 promoter expression vector pET3a (18), which had been digested to completion with NdeI and BamHI. The HAP1h expression vector this created was named pXC53. The structure of pXC53 was confirmed by restriction and

DNA sequence analysis. Plasmids pXC49 and pXC53 were constructed as part of a larger study of HAP1h expression in *Escherichia coli* and will be described in more detail elsewhere.

**Enzyme Expression and Purification**—Human AP endo was expressed in *E. coli* strain BL21/DE3pLysS from pXC53 carrying the HAP1h gene. After induction with isopropyl-1-thio- $\beta$ -D-galactopyranoside for 2 h, cells from a 2-liter culture were lysed in Buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 500 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 1.0  $\mu\text{g/ml}$  pepstatin, and 1 mM dithiothreitol by means of sonic disruption at 0–1 °C. After removal of cell debris by centrifugation at 27,000  $\times g$  for 15 min, the supernatant fraction was adjusted to 100 mM NaCl by addition of Buffer A containing protease inhibitors and dithiothreitol. This was passed over a Q-Sepharose column (100 ml bed volume) connected in series to an S-Sepharose column of the same size. After washing with Buffer A containing 100 mM NaCl, the S-Sepharose column was developed with increasing concentrations of NaCl up to 1 M. AP endo, eluting at high salt (>500 mM NaCl), was further purified by chromatography over a Mono S column by FPLC (Pharmacia Biotech Inc.). The purified enzyme, containing no measurable nuclease activity with double-stranded DNA substrate, eluted from the Mono S column at approximately 450 mM NaCl. The entire purification could be achieved in 2 days with a yield of greater than 6 mg of pure enzyme/liter of *E. coli* culture. Enzyme concentration was determined from Bradford assays (19), which had been calibrated by amino acid analysis. The ratio of protein concentration determined by amino acid analysis to that determined by the Bradford assay was 0.37. Amino-terminal sequencing of the purified enzyme indicated a single protein beginning with Pro<sup>2</sup> of the human AP endo open reading frame. Truncation of the natural Met<sup>1</sup> has also been found for many other *E. coli* recombinant DNA enzymes.

## RESULTS AND DISCUSSION

**Abasic Site Cleavage Products**—The AP site in DNA exists as an equilibrium mixture of the open-chain aldehyde, the  $\alpha$ - and  $\beta$ -hemiacetals, and the open-chain hydrate. Although open-chain aldehydes constitute a very small percentage of total AP sites, they are subject to  $\beta$ -elimination at higher temperatures and in the presence of a variety of agents including common buffers (8, 9, 20) (Fig. 1, *form III*). The  $\beta$ -elimination product itself can be further shortened to the  $\beta,\delta$ -elimination product if strong base is also present during heat exposure (Fig. 1, *form II*).  $\beta$ -Elimination is prevented, however, by  $\text{NaBH}_4$  reduction of the open aldehyde to deoxyribose. Beginning with a 49-bp oligodeoxynucleotide substrate, we examined various potential phosphodiester backbone cleavage products. Fig. 2A illustrates the difference in electrophoretic mobility of the form III  $\beta$ -elimination cleavage product created by treating the unstabilized 49-bp abasic site-containing substrate molecule at 70 °C (*lane 10*), the form II product created by treatment with both heat and base (*lane 9*), and the enzymatically generated form I 20-mer with a free 3'-hydroxyl group at its terminus (*lanes 6–8*). Heat treatment of the stabilized substrate did not alter the substrate (*lane 12*), while treatment of the stabilized 49-bp abasic site-containing substrate with both heat and strong base resulted in a product with mobility of form I 20-mer containing the 3'-hydroxyl group at its terminus (*lane 11*). This latter product has been proposed to involve a 3',5' cyclization intermediate (21).

Comparison of human AP endo activity with substrates generated in the presence or absence of  $\text{NaBH}_4$  is also shown in Fig. 2 (*lanes 1–8*). The rate of the cleavage reaction was faster with the stabilized substrate, and products other than the anticipated form I 20-mer were not detected (Fig. 2). While the absence of undesired spontaneous  $\beta$ -elimination products was not a surprise for the  $\text{NaBH}_4$ -treated substrate, an enhancement of the catalytic rate was unexpected.

**Steady-state Kinetic Parameters**—With the stabilized substrate, formation of form I product was linear for 60 s at an enzyme concentration of 0.02 nM. The apparent  $K_m$  and  $k_{\text{cat}}$  were 100 nM and 10  $\text{s}^{-1}$ , respectively (Fig. 3A and Table I). Enzyme activity required divalent cation ( $\text{Mg}^{2+}$ ) and was com-

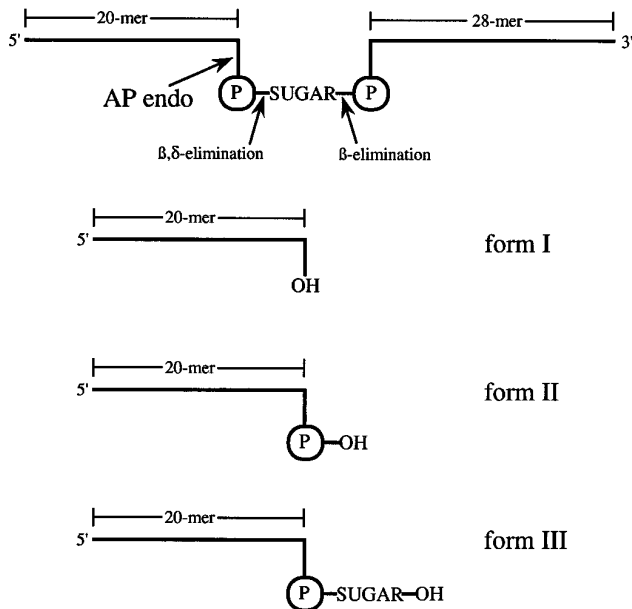


FIG. 1. Predominant cleavage positions in a 49-mer oligonucleotide containing an abasic site at position 21. For clarity, only the strand containing the abasic site is depicted. The site of enzymatic AP endo cleavage 5' to the abasic site is contrasted with the site where  $\beta$ -elimination occurs 3' to the abasic site and the site where  $\delta$ -elimination occurs. *Forms I, II, and III* show the products relative to the 5'-end of the oligomer. *Form I* results from nicking by AP endo, which leaves a free 3'-hydroxyl group at the end of the 20-mer. *Form II* is the product after  $\beta,\delta$ -elimination and results in a phosphate group at the end of the 20-mer oligonucleotide. *Form III* is the product of  $\beta$ -elimination where the phosphodeoxyribose remains attached to the 3'-end of the 20-mer.

pletely blocked by 4 mM EDTA in the absence of added  $Mg^{2+}$ .

To understand the observed rate enhancement with the stabilized substrate, the spontaneous  $\beta$ -elimination product (Fig. 1, *form III*) was deliberately generated by heating unstabilized substrate without enzyme for 30 min at 70 °C. When this HDP was included in the reaction mix, it proved to be an inhibitor of AP endo activity (Fig. 3). When inhibition was studied as a function of stabilized substrate concentration, HDP increased the apparent  $K_m$  for stabilized substrate without markedly altering  $V_{max}$  (i.e. ordinate-intercept; Fig. 3A). From Dixon analysis of the inhibition, the  $K_i$  for this competitive inhibitor was 30 nM (Fig. 3B). Thus the enhancement of AP endo activity for reactions in which the abasic site DNA substrate was stabilized by  $NaBH_4$  reduction was probably due to the absence of spontaneously generated HDP, which could have accumulated in the reaction mixture. This proposal is consistent with the degree of inhibition observed with spontaneously generated HDP (80%, Fig. 2B) and that determined here with deliberately generated HDP (Fig. 3), where inhibition under the conditions described in Fig. 2 would be expected to be 60%.

**Substrate Binding**—To assign the stoichiometry of binding of AP endo and DNA, we used an electrophoretic mobility shift assay to measure the relative amount of enzyme-DNA complex formed at various enzyme/DNA ratios (Fig. 4). There is more complex formed with an abasic site-containing oligomer than with a double-stranded oligomer without an abasic site, indicating that binding to substrate or nicked DNA was much tighter than binding to "intact" double-stranded DNA. The apparent equilibrium dissociation constant for the "abasic site" DNA was less than 0.1 nM. The enzyme concentration saturating the abasic site-containing DNA corresponded to a molecular ratio of 1 enzyme to 1 DNA molecule (Fig. 4B).

To delineate the individual components of substrate DNA binding that are embedded within the Michaelis constant ( $K_m$ ),

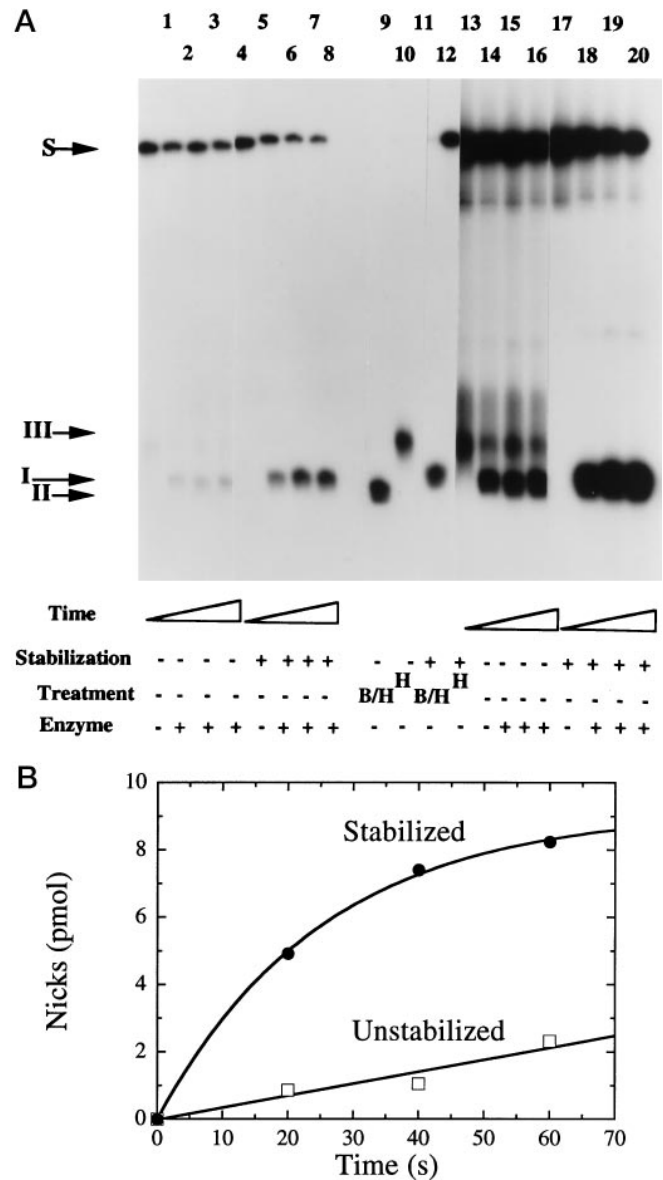


FIG. 2. Stabilization of the abasic site enhances the rate of AP endo activity. A, an abasic site was created in the absence (lanes 1–4, 9, 10, and 13–16) or presence (lanes 5–8, 11, 12, and 17–20) of 0.1 M  $NaBH_4$  as described under "Experimental Procedures." The reaction was initiated by the addition of enzyme to a mixture containing 2.3  $\mu M$  49-bp substrate DNA (S) with an abasic site at position 21. The reaction was terminated at 0, 20, 40, and 60 s (shown as increasing time) by the addition of gel loading dyes. Lanes 13–20 represent a longer exposure of the same autoradiogram shown in lanes 1–8 in order to demonstrate the presence of HDP more clearly. Lanes 10 and 12 were heated (H) to 70 °C for 30 min before the addition of loading dyes; lanes 9 and 11 were heated under the same conditions, except that NaOH was added to a final concentration of 0.4 mM (B/H). The solution was neutralized with 2 M HCl before the addition of loading dyes. The positions of the form I, II, and III products are indicated (I–III). B, time course of incision product formation (form I) was quantified by PhosphorImager analysis of the gel depicted in A.

we measured substrate association and dissociation with AP endo by using a challenger to trap free enzyme, in order to estimate the concentration of enzyme-substrate complex. In the presence of challenger, enzyme that is bound productively to substrate can nick DNA, whereas free enzyme is quenched. Thus, activity is proportional to the concentration of ES complex. Various agents including single-stranded DNA, oligo(dT), higher concentrations of unlabeled abasic site-containing substrate, heparin, and HDP were evaluated for their ability to

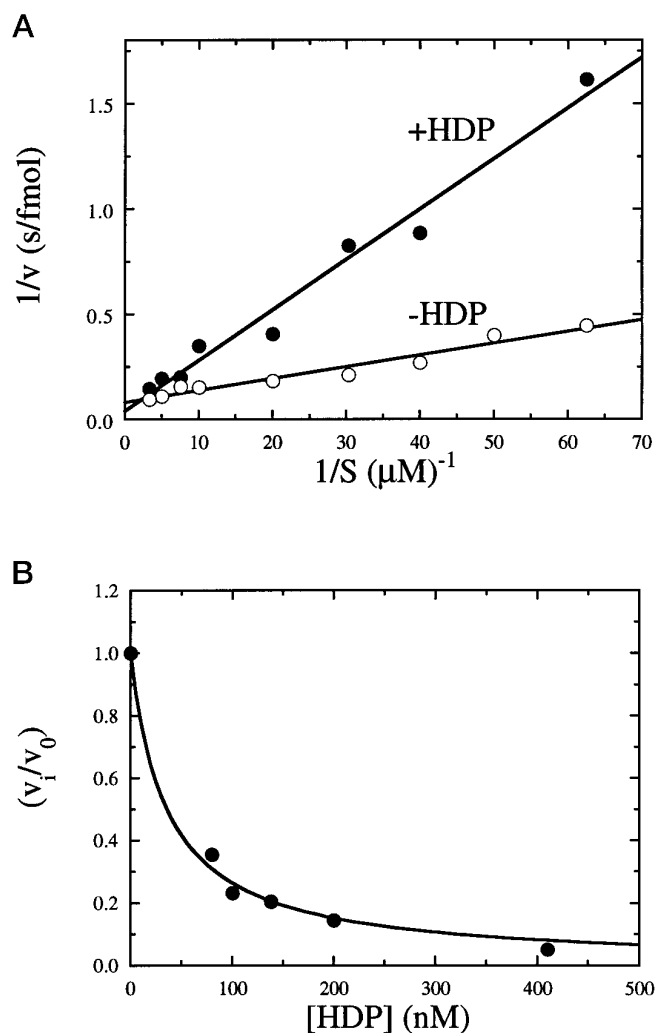


FIG. 3. Inhibition of AP endo by substrate heat degradation product. The HDP was generated as described under "Experimental Procedures." A, double-reciprocal plot illustrating the relationship between AP endo initial velocity and abasic DNA substrate concentration in the absence (○) and presence (●) of 165 nM HDP. The concentration of AP endo was 0.02 nM, and 20-mer product formation was determined after 30 s. B, Dixon plot of HDP inhibition of AP endo activity. The rate of formation of 20-mer product by 0.4 nM AP endo was determined over a 30-s interval at different HDP concentrations. The concentration of substrate (5'-end labeled abasic 49-mer) was 33 nM. Relative initial velocities ( $v_1/v_0$ ) were fitted to an equation for competitive inhibition (solid line), and the fitted  $K_i$  was 33 nM.

TABLE I  
Comparison of observed and calculated steady-state kinetic parameters for human AP endonuclease cleavage of a 49-base pair oligonucleotide with a single abasic site

	Steady-state kinetic parameter		
	$K_m$	$k_{cat}$	$k_{cat}/K_m$
	nM	$s^{-1}$	$M^{-1} s^{-1}$
Observed value <sup>a</sup>	100	10	$1.0 \times 10^8$
Calculated value <sup>b</sup>	200	10	$0.5 \times 10^8$

<sup>a</sup> Taken from Fig. 3A.

<sup>b</sup> Assuming the mechanism depicted in Scheme I,  $K_m = (k_{off} + k_{cat})/k_{on}$ , where  $k_2$  is equivalent to  $k_{cat}$ . The association and dissociation rate constants were  $5 \times 10^7 M^{-1} s^{-1}$  and  $0.04 s^{-1}$  as derived from the experiment in Fig. 5. The calculated values were confirmed by kinetic simulation.

trap free enzyme and prevent catalytic cycling. An effective trap should quench catalytic cycling when preincubated with enzyme and substrate. Single-stranded DNA, oligo(dT), and unlabeled abasic site-containing substrate were ineffective

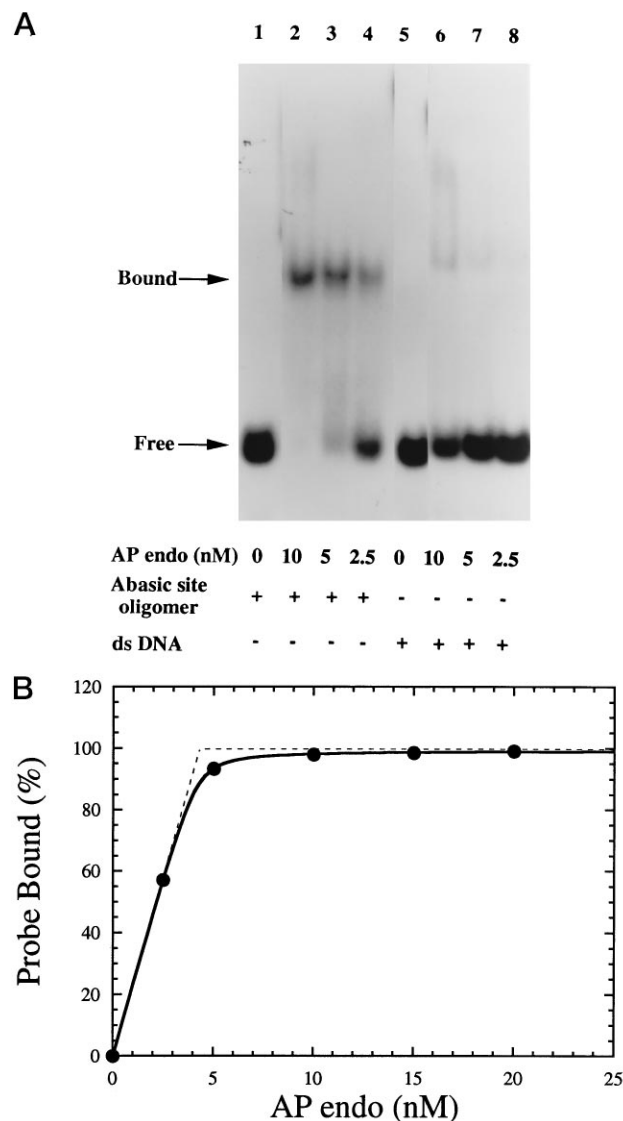


FIG. 4. Binding of AP endo to an oligomer with or without an abasic site. A, an electrophoretic mobility shift assay was performed as described under "Experimental Procedures" to measure complex formation. AP endo, at the indicated concentrations, was allowed to bind to 5 nM oligomer with or without an abasic site before separation of enzyme-DNA complex (Bound) from free DNA (Free). ds DNA, double-stranded DNA. B, quantitation of bound and free DNA. The results from the experiment shown in panel A were quantified by PhosphorImager analysis. The data were fitted to the quadratic equation by nonlinear least squares methods (solid line) and indicated that AP endo binding to DNA with an abasic site was very tight. The inflection point of the binding isotherm (*i.e.* 4.3 nM) reflects the concentration of DNA where the ratio of enzyme/DNA is 1.

traps. Unlabeled abasic site-containing substrate probably was not a good trapping agent, since it is an excellent substrate for human AP endonuclease as suggested by the high turnover number and low  $K_m$ . Accordingly, attempts to use unlabeled substrate resulted in depletion of both labeled and unlabeled substrate at the concentration of enzyme used to perform single-turnover experiments. However, of particular interest were the results with HDP and heparin. HDP or heparin alone were ineffective in completely preventing cycling of the enzyme. However, in the presence of both heparin and HDP, the enzyme was prevented from catalytic cycling over a 10-s interval (data not shown). In addition, the amount of product formed per catalytic cycle was directly proportional to enzyme concentration. Thus, the combination of heparin and HDP effectively competes for substrate binding without being "consumed,"

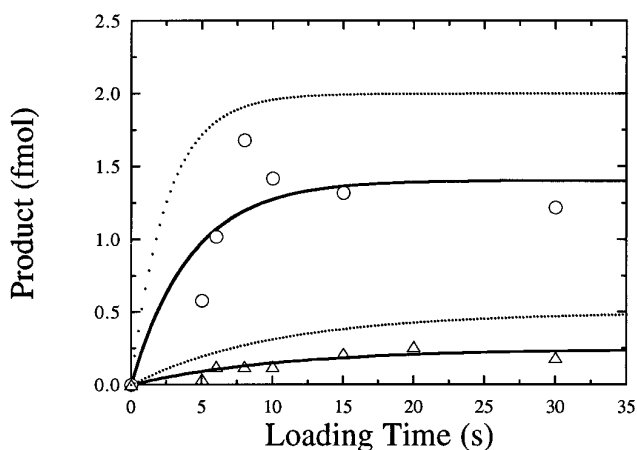


FIG. 5. Association of AP endo and oligonucleotide with an abasic site. AP endo, 0.1 ( $\Delta$ ) or 0.4 nM ( $\circ$ ), was mixed with a 10-fold greater concentration of 49-mer oligonucleotide containing an abasic site. At time intervals after mixing, aliquots were removed and added to a mixture of  $Mg^{2+}$  (10 mM final concentration) and trap (6.1  $\mu$ M HDP and 2 mg/ml heparin, final concentration) in a total volume of 5  $\mu$ l. The reactions were terminated after 10 s, and the amount of product 20-mer formed was determined as described under "Experimental Procedures." Data were fitted to an ascending exponential (solid line) to derive apparent first-order rate constants. The first-order rate constants were  $0.09 \pm 0.04$  and  $0.24 \pm 0.14$   $s^{-1}$  for 0.1 and 0.4 nM enzyme, respectively. Additionally, substrate binding time courses that were generated from kinetic simulation (dotted lines), using  $k_{cat}/K_m$  as the apparent rate association constant and assuming an irreversible binding reaction, demonstrate that there is good agreement with the binding time courses determined by the challenge assay.

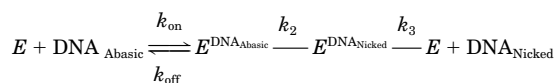
making this mixture a better trap than unlabeled substrate.

The association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) rate constants for the AP endo-substrate interaction were determined by examining the time dependence of ES formation by employing the challenge assay as described above. To follow ES formation, enzyme and substrate were incubated under pseudo-first order conditions in the absence of  $Mg^{2+}$  for periods up to 30 s (Fig. 5). For each period, the amount of ES complex was measured by initiating enzymatic activity with  $Mg^{2+}$  and trap. Substrate was able to bind to AP endo in a concentration-dependent manner in the absence of  $Mg^{2+}$ . The apparent half-time ( $t_{1/2}$ ) for enzyme and DNA association was 7.4 and 2.9 s for 0.1 nM and 0.4 nM enzyme, respectively, where  $t_{1/2}$  is  $\ln 2/k_{obs}$ . Assuming a simple binding model ( $E + S \rightleftharpoons ES$ ), these half-times correspond to an apparent association rate constant of  $5 \times 10^7$   $M^{-1}$   $s^{-1}$  and suggest that the dissociation rate constant is very slow ( $k_{off} \approx 0.04$   $s^{-1}$ ). The corresponding equilibrium dissociation constant is, therefore, 0.8 nM. Longer incubations (*i.e.* 5 min) with several enzyme and substrate concentrations (1–10 nM) resulted in an equilibrium level of complex formation consistent with this dissociation constant. This equilibrium constant is larger than that determined by electrophoretic mobility shift assay (Fig. 3). Therefore, the equilibrium constant determined by electrophoretic mobility shift assay appeared to substantially overestimate DNA binding affinity.

**Rate-limiting Step**—When product release is slower than chemistry, the time course of product formation is biphasic. There is an initial burst of product formation equivalent to the active enzyme fraction, followed by a linear phase that represents slow product dissociation. However, the rapid consumption of substrate in the presence of high enzyme concentrations precluded accurate estimates of product formation at times accessible by manual mixing and sampling techniques. Results of Wilson *et al.* (22) suggest that chemistry of the enzymatic reaction is itself rate-limiting. In their study, the presence of a 5'-phosphorothioate derivative at the scissile bond phosphate

substantially reduced the rate of the enzymatic reaction, an observation that is consistent with an elemental effect on the nicking reaction. These results indicate that chemical cleavage or a conformational change prior to nicking is rate-limiting, instead of a subsequent step such as product release. If steps occurring after chemistry are so rapid that they are not rate-limiting, the ratio of  $k_{cat}/K_m$  is a measure of the apparent second order rate constant for productive substrate binding. In keeping with this idea, it is noted that the values for  $k_{cat}/K_m$  and the association rate constant for AP endo are comparable (Table I). Additionally, kinetic simulation of substrate binding from these steady-state measurements (Fig. 5, dotted lines), assuming an irreversible binding reaction, demonstrate that there is good agreement with the binding time courses determined by the challenge assay (Fig. 5, solid lines). The amplitude of the simulated time courses is reduced to that observed for the time courses of complex formation determined with a trapping agent, if a dissociation rate constant of 0.10  $s^{-1}$  is included (data not shown).

**Kinetic Mechanism**—The results described above can be used to derive a minimal kinetic scheme consistent with a Briggs-Haldane mechanism (Scheme I). Additionally, steady-state kinetic parameters can be calculated or derived from simulation of the model depicted in Scheme I for comparison to observed values (Table I). In this scheme, product release (*i.e.*  $k_3$ ) is assumed to be rapid since a phosphorothioelemental effect has been reported (22), and  $k_{cat}$  reflects the chemical step (*i.e.*  $k_2$ ).



SCHEME I

**Concluding Remarks**—In this study, we used steady-state and transient-state kinetic parameters to describe human AP endo enzymatic nicking of a synthetic oligomer with a single abasic site. This study was facilitated by the observation that the spontaneous heat degradation product of abasic site-containing DNA inhibits the enzyme competitively. The minimal mechanism proposed is a simple Briggs-Haldane scheme, where the forward catalytic reaction competes with the dissociation of the enzyme-DNA complex. This kinetic mechanism is a prerequisite for studies of the kinetic properties of mutant or altered enzyme forms and will facilitate identification of chemotherapeutic agents designed to inhibit DNA repair. Definitive evidence is lacking that AP endo participates in base excision repair. However, since the turnover number for DNA polymerase  $\beta$ , known to be integral in the single-nucleotide base excision repair pathway (13), is approximately 1  $s^{-1}$  (23, 24), AP endo would not be rate-limiting if operating under optimal conditions. It remains to be determined if human AP endo enzyme activity can be modulated *in vivo* and if this will translate to altered sensitivity to DNA damaging agents repaired by base excision repair.

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