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## Role of Active Site Tyrosines in Dynamic Aspects of DNA Binding by AP endonuclease

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### Abstract

AP endonuclease (AP endo), a key enzyme in repair of abasic sites in DNA, makes a single nick 5' to the phosphodeoxyribose of an abasic site (AP-site). We recently proposed a novel mechanism, whereby the enzyme uses a key tyrosine (Tyr<sup>171</sup>) to directly attack the scissile phosphate of the AP-site. We showed that loss of the tyrosyl hydroxyl from Tyr<sup>171</sup> resulted in dramatic diminution in enzymatic efficiency. Here we extend the previous work to compare binding/recognition of AP endo to oligomeric DNA with and without an AP-site by wild type enzyme and several tyrosine mutants including Tyr<sup>128</sup>, Tyr<sup>171</sup> and Tyr<sup>269</sup>. We used single turnover and electrophoretic mobility shift assays. As expected, binding to DNA with an AP-site is more efficient than binding to DNA without one. Unlike catalytic cleavage by AP endo, which requires both hydroxyl and aromatic moieties of Tyr<sup>171</sup>, the ability to bind DNA efficiently without an AP-site is independent of an aromatic moiety at position 171. However, the ability to *discriminate* efficiently between DNA with and without an AP-site requires tyrosine at position 171. Thus, AP endo requires a tyrosine at the active site for the properties that enable it to behave as an efficient, processive endonuclease.

### Keywords

AP endonuclease; APE1; Apex; base excision repair; kinetics; EMSA; tyrosine; DNA binding

### Introduction

Abasic (AP) sites are major lesions in DNA generated either spontaneously or through the action of endogenous or exogenous factors (8,13,23,24). Up to 200,000 abasic sites are created in a cell each day (13,23,36). Unrepaired abasic sites can lead to further DNA damage in the form of ds breaks when there are two or more sites within close proximity to each other on opposite strands (6,32), as well as inhibition of topoisomerases (48), replication and transcription (52). To correct these lesions, living systems have evolved abasic site repair (ASR) (44). One of the key enzymes in ASR is apurinic/aprimidinic endonuclease (AP endo, Apex, HAP1, Ref-1, Ape) (9,10,17,25,40,42,43), which nicks the DNA backbone immediately 5' to the phosphodeoxyribose (dRP) of the AP-site. The resulting dRP is then removed by a lyase activity, usually that of DNA polymerase- $\beta$ , generating a single nucleotide gap that is filled by a DNA polymerase. Once the correct nucleotide is inserted, the nick is sealed by a ligase (10,13,25,44).

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AP endo was first identified as having a role in DNA repair when *Escherichia coli* mutants deficient in exonuclease III, the prokaryotic equivalent to AP endo, were shown to have an increased sensitivity to alkylating agents but no marked sensitivity to UV or  $\gamma$ -irradiation (51). Mammalian AP endo, initially isolated from human tissue (19,26,34) and cell lines (4, 21,22), was confirmed as the *E. coli* equivalent because of its enzymatic activity and because the gene complemented *E. coli* deficient in exonuclease III for resistance to alkylating agents (9,40,42). AP endo binds abasic (AP) site-containing DNA in the absence of divalent cation with remarkable affinity as shown using an electrophoretic mobility shift assay (EMSA) (11, 28,29,43,47) and single turnover (ST) kinetics (43). As a processive enzyme (5), AP endo also binds DNA lacking an AP-site with surprising affinity (2,43). Indeed, the fact that the enzyme is processive (5) requires that AP endo bind DNA, whether or not it contains an AP-site, discriminate normal DNA from AP-site-containing DNA, and, once an AP-site is cleaved, move on quickly to locate the next AP-site. In this paper we have used two methodologies to generate a more complete picture of how AP endo recognizes DNA with and without an AP-site. EMSA is routinely employed to measure DNA binding with proteins that interact with a particular consensus sequence (15,18,39,46,49,50) or with a particular lesion (11,14,29,47), while single turnover and steady state kinetics examine binding from the viewpoint of catalytic activity.

## Materials and Methods

### Source of WT and mutant AP endo

Human AP endo was obtained by expression of the gene cloned into plasmid pXC53 and purified as described earlier (43). All mutations were made starting with this plasmid. Preparation of Y128A, Y171A and Y269A is described in Mundle et al. (35). Y171F and Y171H were initially the gift of Dr. David Wilson III, although the Y171F mutation was subsequently created in the pXC53 plasmid. To ensure consistency of the two mutants from the Wilson laboratory with others prepared in the Strauss laboratory, data from WT AP endo from the Wilson laboratory were shown to match that generated with WT from the Strauss laboratory (data not shown).

### Preparation of substrate

Substrate lacking an AP-site was the 45-mer containing a U at position 21 shown below and used in previous studies (27,35). Substrate containing an AP-site originated with the same 45-mer oligonucleotide:

5'-AGC TAC CAT GCC TGC ACG AAU TAA GCA ATT CGT AAT CAT GGT CAT - 3'

3'-TCG ATG GTA CGG ACG TGC TTG ATT CGT TAA GCA TTA GTA CCA GTA - 5'

The 5' end of the U-containing strand was labeled with T4 polynucleotide kinase, (New England Biolabs, Beverly, MA) as described earlier. The AP-site was generated through digestion for 30 min at 37 °C with *E. coli* uracil-DNA glycosylase (UNG) (1U/100 pmol of U-containing oligonucleotide; Epicenter Technologies, Madison, WI) in the presence of 0.2 M NaBH<sub>4</sub>. NaBH<sub>4</sub> reduces and stabilizes the AP-site so that no  $\beta$ elimination product forms (43). NaBH<sub>4</sub> does not interfere with the glycosylase because UNG has no lyase activity (7, 30,31). Furthermore, the  $\beta$ -elimination product of an AP-site inhibits enzymatic activity (43). After UNG was inactivated at 70 °C – 75 °C for 5 min, the solution was slow cooled to 22 °C.

## EMSA binding assays

Substrate (0.25 nM) was incubated in the presence of enzyme (0–250 nM) at 25 °C for 30 min. Incubation medium contained 0.5% polyvinyl alcohol, 10% glycerol, 100 µg/ml bovine serum albumin, 6–8 mM NaCl, and 0.1 mM EDTA in 50 mM HEPES-NaOH, pH 7.5. Bound and free oligonucleotide were resolved at 70 V on 8% native polyacrylamide gels in 1X tris/borate/EDTA buffer (41) at 4 °C and quantified by PhosphorImager analysis. Substrate incubated in the absence of enzyme was used as the control in each case. Bound DNA was calculated as the difference between unbound DNA in the absence and presence of protein. The  $K_d$  value was calculated at the concentration at which half maximal binding was observed from the following relationship (12):

$$K_d = [E]_{1/2}[S]_{1/2} / [ES]_{1/2} \quad \text{Equation 1}$$

When the enzyme concentration was much higher than the substrate concentration and the bound enzyme did not lead to appreciable decrease in free enzyme concentration, the equation reduced to:

$$K_d = [E]_{1/2} \quad \text{Equation 2}$$

## Single turnover assays

ST assays to determine substrate binding were performed as described previously (43). In brief, substrate was permitted to bind with enzyme at the concentrations indicated in the presence of 4 mM EDTA to prevent cleavage of substrate during binding. At the indicated times cleavage was initiated by addition of 10 mM MgCl<sub>2</sub> in the presence of trap (2 mg/ml heparin and 6.1 µM HDP) (see ref.(43)), which prevents additional rounds of catalysis. Reactions were terminated by addition of 0.5 M EDTA to a final concentration of 87 mM. Substrate and product were resolved by denaturing gel electrophoresis and quantitated by PhosphorImager analysis (43). The presence of slope = 0 after binding reached equilibrium indicated that conditions for ST were met. While ST methodology provides parameters that more accurately describe the kinetics of enzyme action (43), single turnover methodology is no longer appropriate for mutants where  $k_{-1}$  exceeds  $k_{cat}$  (12). For such mutants, the concentration of ES at equilibrium is underestimated and results in a corresponding overestimate in  $K_d$  value.

The  $K_m$  for enzymes that follow Briggs-Haldane kinetics is described as  $K_m = (k_{-1} + k_{cat}) / k_{+1}$ . When  $k_{cat} \ll k_{-1}$ , the equation reduces to  $K_m = k_{-1} / k_{+1}$ , that is, the  $K_m$  and  $K_d$  values are similar. In fact, for the Tyr<sup>171</sup> mutant series, the  $k_{cat}$  values for the Tyr<sup>171</sup> mutant series are so slow ( $10^{-3}$ – $10^{-4}$  s<sup>-1</sup>) (35) that the  $K_d$  is essentially the same as the  $K_m$ , measured under steady state conditions. Experiments with Y269A were carried out with 0.2 nM enzyme and 4 nM substrate (final concentration), 0.4 nM enzyme and 4 nM substrate, and 1 nM enzyme and 4 nM substrate. Binding by Y128A was measured at 4 nM enzyme and 4 nM substrate, while binding by Y171A was measured at 7 nM enzyme and 7 nM substrate; binding by Y171F was examined at 4 nM, 10 nM or 20 nM enzyme and 4 nM substrate; and binding by Y171H was examined at 4 nM, 20 nM or 40 nM enzyme and 4 nM substrate. Previously published binding experiments for WT enzyme were performed at 0.1 nM enzyme and 1 nM substrate, 0.4 nM enzyme and 4 nM substrate and 1 nM enzyme and 10 nM substrate (43). For the current series of experiments, binding for WT enzyme was repeated at 0.4 nM enzyme and 4 nM substrate. WT and Y269A enzymes were allowed 20 s to complete a single round of catalysis after addition of Mg<sup>2+</sup>/trap. For Y128A, 30 s was used and for the Tyr<sup>171</sup> mutants, either 20 s or 7200 s was used with no difference in results. During the binding step, the concentration of salt was 68 mM, while during the catalytic step, the concentration of salt was 40 mM NaCl. This salt concentration was sufficient to ensure cleavage of substrate previously bound to enzyme. For each enzyme 3–5 experiments were performed.

Single turnover  $K_d$  values were calculated from the relationship:

$$K_d = [E_i - ES_e][S_i - ES_e] / ES_e \quad \text{Equation 3}$$

where  $E_i$  and  $S_i$  are initial concentrations of enzyme and substrate respectively and  $ES_e$  is the concentration of ES at equilibrium. Substrate association and dissociation constants were calculated explicitly from the relationship (12):

$$k_{obs} = k_{+1}[S] + k_{-1}, \quad \text{Equation 4}$$

where  $k_{obs} = 0.693/t_{1/2}$  was determined experimentally.

## Results

### $K_d$ values determined by ST/SS

In order to compare binding of AP endo to DNA in a quantitative fashion, we employed single turnover (ST), steady state (SS) and EMSA methodologies. ST methodology allowed substrate containing a single AP-site to bind reversibly to enzyme under conditions where there was no conversion to product (12,43). Divalent cation was then added to convert all ES to product, while the presence of a trap prevented the enzyme from initiating a second round of catalysis. Therefore, the amount of cleavage product directly reflected the amount of ES at equilibrium. We originally established ST conditions in order to describe the behavior of WT AP endo in recognizing and cleaving an AP-site (43) and showed that the  $K_d$  for WT enzyme was 0.8 nM. Using a 45-mer oligonucleotide with an AP-site at position 21, we compared the time course of binding by WT and five tyrosine mutants (Figures 1 and 2 and Table 1).

We considered that two tyrosines, Tyr<sup>128</sup> and Tyr<sup>269</sup>, located outside the active site, were likely to be involved in DNA binding. These residues interact with AP-site containing oligonucleotide just upstream and downstream of the AP-site in the co-crystal structure (33). Mutation of either of these residues to alanine results in loss of one-two orders of magnitude in enzymatic efficiency (35). The  $K_d$  value for Y128A (Table 1) was 52±22 nM (SE,3). Thus, converting Tyr<sup>128</sup> to alanine resulted in a 65-fold increase in  $K_d$  in comparison to that of WT. Conversion of Tyr<sup>269</sup> to alanine resulted in an increased  $K_d$  to 7±0.5 nM (SE,3), an increase of 9-fold over that of the WT enzyme. Equation 4 and the time courses presented in Figure 1 allowed us to determine explicit substrate association and dissociation constants (12) for Y128A and Y269A. Values for Y128A were  $5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$  and  $0.2 \text{ s}^{-1}$  respectively. Similarly, the values for Y269A were  $2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  and  $0.2 \text{ s}^{-1}$  respectively. Thus, the rate of substrate association was decreased 10–20-fold from that of wild type, while the rate of substrate dissociation was increased about 5-fold for both mutants. These values indicated that the Briggs-Haldane relationship, where  $K_m = (k_{-1} + k_{cat})/k_{+1}$ , was conserved for Y128A and Y269A. Also, these data confirmed that the two residues were involved in binding and recognition of substrate.

Tyr<sup>171</sup> is located in the active site of AP endo (33). Conversion of Tyr<sup>171</sup> to phenylalanine or alanine results in major loss (four-five orders of magnitude) in enzymatic efficiency (35) See Table 1. Mutants at Tyr<sup>171</sup> bound substrate very poorly, when examined by ST methods:  $[ES]_{eq}$  values were extremely low. When Equation 3 was used to calculate  $K_d$  values from  $[ES]_{eq}$ , the values far exceeded those of the other mutants [2,900 ±676 (SE,4) for Y171A; 5,900 ±1927 (SE,4) for Y171F and 61,585 ±19,230 (SE,3) for Y171H]. However, these values were not valid indicators of  $K_d$ , because the  $k_{cat} \ll k_{-1}$ . Because ES would dissociate before it could be detected by this method, attempts to measure binding based on ST methods could not provide accurate values for these mutants. Nevertheless, we were able to obtain a valid  $K_d$  measurement, because the Briggs-Haldane relationship reduced to  $K_m = k_{-1}/k_{+1}$  (Table 1, column 6) under these conditions. From these comparisons, loss of the hydroxyl

group alone (Y171F) or the entire phenolate (Y171A) increased  $K_d$  by 318–450 fold. Adding a positive charge (Y171H) drastically interfered with binding and recognition and increased  $K_d$  by 2500-fold.

Although it was inappropriate to use the observed  $[ES]_{eq}$  obtained by SS measurements for calculating kinetic constants, the shape of the single turnover binding curves allowed us to obtain valid  $t_{1/2}$  and  $k_{obs}$  values, using Equation 4 for Y171F. Since  $k_{-1} = K_m k_{+1}$ , one could then calculate theoretical values for  $k_{+1}$  and  $k_{-1}$ . These values were  $10^5 \text{ M}^{-1}\text{s}^{-1}$  and 0.03 for  $k_{+1}$  and  $k_{-1}$  respectively for Y171F. The  $k_{+1}$  value was three orders of magnitude below that of the WT enzyme, corroborating the supposition that  $k_{cat} \ll k_{-1}$ , while the  $k_{-1}$  value did not change. Thus, the rate of substrate binding was  $\sim 3$  orders of magnitude lower than that by WT; once bound, substrate dissociated at  $\sim$ same or somewhat faster rate. This result indicated that the Y171F recognized the AP-site poorly. Similar calculations for Y171H failed, because we were unable to obtain an explicit  $K_m$  (the enzyme failed to saturate at or below  $1.3 \mu\text{M}$  (35)). Since the inflection point on the binding curve for Y171A occurred too quickly to be detected under the conditions used here (Figure 2), we could not obtain an explicit value for  $t_{1/2}$  so that similar calculations for Y171A also failed.

### **$K_d$ determined by EMSA**

In order to compare binding of the various tyrosine mutants to DNA lacking an AP-site, we turned to a method that did not require enzymatic activity. We examined the ability of the WT enzyme and the tyrosine mutants to discriminate between substrate with or without a single AP-site at position 21 by EMSA. In order to perform the studies in a quantitative fashion so that  $K_d$  values could be compared with data derived from ST or steady state studies as appropriate, the concentration of AP endo was varied between 0 and 300 nM, while the concentration of substrate was held constant at 0.25 nM.  $K_d$  values were then calculated using either Equation 1 or Equation 2.

Figure 3 represents an example of an EMSA performed with WT and Y171A proteins. In all cases, enzyme bound to DNA (ES), determined by the degree of band retardation, was dependent on the concentration of protein. In this case, the concentration of protein at which maximum band retardation was reached was  $\sim 25$  nM for WT and  $\sim 250$  nM for Y171A. Under these conditions  $< 6\%$  of initial substrate was converted to product when WT enzyme was incubated with substrate under EMSA conditions, ensuring that most gel-shifted oligonucleotide was not converted to product (data not shown). This result was expected, because 4 mM EDTA present in the incubation mix prevents AP-site cleavage by enzyme (43).

Even though the molecular weights of WT and individual mutant proteins were essentially the same, the mobility of ES depended on the mutation. For instance, the mobility of the major species of ES of Y171F and Y171A was the same as that of WT. However, the mobility of the major ES complex of DNA with Y128A was greater than that of WT, while the mobility of the major ES complex of DNA with Y171H and Y269A was less than that of WT (data not shown). Although the changes in mobility might tempt one to presume that the ES complex of DNA with one mutant or the other was more or less stable than that of DNA with WT, the quantitative data presented below and above do not support this possibility.

Figure 4 and Table 2 compare the concentration dependence of binding behavior of mutants with that of WT enzyme, using an oligonucleotide with or without an AP-site. As in the case of ST measurements, each mutant provided a unique binding pattern. Table 2 presents the experimental results comparing  $K_d$  values determined by EMSA for substrate with and without an AP-site. Binding of a substrate with an AP-site followed the expected hyperbolic pattern with saturation occurring at different concentration of protein for the different mutants. None



of the mutants increased the  $K_d$  obtained by EMSA analysis more than 18-fold in comparison with that of WT (Table 2) or reflected previously shown changes in enzymatic efficiency (See Table 1). EMSA-derived values also could not be used to obtain substrate association/dissociation constants in the standard equations used for kinetic analysis. Nevertheless, EMSA analysis of binding of substrate with an AP-site provided  $K_d$  values for WT, Y269A and Y128A that generally agreed with or were somewhat higher than those from single turnover studies (43). On the other hand, EMSA greatly underestimated the  $K_d$  values for all the Tyr<sup>171</sup> mutants as shown by comparison with  $K_d$  values obtained from SS and ST experiments.

We then compared the ability of WT and Tyr<sup>171</sup> mutant enzymes to bind the same ds oligomer in which the AP-site was replaced with a uridine residue (Figure 4B). Since there was no AP-site, it was not possible to obtain  $K_m$  or  $k_{cat}$  values or to perform a ST assay. The  $K_d$  values determined by EMSA analysis are presented in Table 2. As expected from previous studies (43), the ability of WT and most mutant enzymes to bind DNA lacking an AP-site was reduced in comparison to DNA binding with an AP-site. Consequently,  $K_d$  values were higher for substrate lacking an AP-site. However, the relative ordering of the  $K_d$  values was informative: the  $K_d$  value for Y171F was *lower* than that of WT enzyme, which implied that Y171F bound more tightly to DNA lacking an AP-site than did WT enzyme. Indeed, aside from Y171F, all the Tyr<sup>171</sup> mutants bound DNA lacking an AP-site *equally* well. Thus, an aromatic residue at position 171 was not required for AP endo to bind non AP-site containing DNA. The hydroxyl of Tyr<sup>171</sup>, required for normal cleavage and recognition of an AP-site, actually *interfered* with binding to DNA without an AP-site. Thus, Tyr<sup>171</sup> can be viewed as enabling the enzyme to scan DNA more efficiently.

Finally the ability to *discriminate* between DNA with and without an AP-site was compared by examining the ratios of the  $K_d$  values obtained by EMSA analysis for DNA with and without an AP-site (Table 2, column 4). Here it became clear that WT AP endo is optimally suited to discriminate between the two forms and that all the Tyr<sup>171</sup> mutants fail in this requirement.

## Discussion

We have examined binding of AP endo to ds DNA with and without an AP-site by two different methods. One involves values generated using kinetic analysis, while the other employs electrophoretic mobility shift assays. Although each method has disadvantages, the two together provide a comprehensive picture of the role of three key tyrosines in recognizing and binding DNA. Tyr<sup>128</sup> and Tyr<sup>269</sup> contribute to endonuclease activity primarily through recognition and binding of DNA. Both are present outside the active site but make contact with substrate DNA (33). On the other hand, Tyr<sup>171</sup> contributes to enzymatic function through direct involvement in catalysis as well as binding and recognition of DNA. Furthermore, Tyr<sup>171</sup> is intimately involved in enabling AP endo to discriminate DNA with an AP-site from DNA without one. The WT enzyme is 17-fold more effective in binding AP-site containing DNA than normal DNA, while the tyrosine mutants discriminate normal and AP-site containing DNA poorly.

Kinetic measurements (ST and SS) require production of product, in this case, a cleaved AP-site. Results obtained by kinetic analysis from ST and SS measurements are useful in establishing a kinetic scheme (27,35,43). As valuable as ST is in dissecting steps in the kinetic scheme, however, this methodology fails when the substrate dissociation constant exceeds the  $k_{cat}$ . Here the ES complex dissociates so quickly that it cannot be detected accurately. In that case we can still use the  $K_m$  value derived through steady state measurements to approximate the  $K_d$ , because the explicit description for  $K_m$  [ $K_m = (k_{-1} + k_2)/k_{+1}$ ] reduces to  $k_{-1}/k_{+1}$ , which is the same as  $K_d$ . The current data are consistent with previously reported changes in enzymatic

activity of the various mutants that enabled us to propose a novel mechanism of action for the enzyme (35).

Unlike the ST and SS data, the changes in  $K_d$  for substrate containing an AP-site obtained by EMSA do not correlate with changes in enzymatic efficiency. Although increases in  $K_d$  measured by EMSA are roughly consistent with decreases in enzymatic efficiency for Y128A and Y269A, (Table 2), the changes observed in the Tyr<sup>171</sup> series are not at all consistent. In the Tyr<sup>171</sup> series, increases in  $K_d$  measured by EMSA range from 1.2-fold in the case of Y171F to 9.3-fold in the cases of Y171A and Y171H. These increases fail to reflect the changes in  $K_m$  or the profound changes in enzymatic efficiency (> four orders of magnitude decrease). Also, the order of change in  $K_d$  values as determined by EMSA implies that the loss of the tyrosyl hydroxyl is hardly significant in interacting with AP-site containing DNA, a conclusion that is not supported by the kinetic data. It is easy to see how one might conclude that tyrosines are not essential for catalysis by AP endo based on EMSA studies, where the  $K_d$  value for Y128A binding is increased even more than that for Y171A (38). The discrepancy between EMSA and kinetic analyses could be due to differences in assay conditions: the physical conditions employed during EMSA analysis, such as shear force and the presence of a voltage differential, do not come into play when catalysis is measured.

Kinetic methods, which require enzymatic cleavage to detect the ES intermediate, also cannot provide information on how well the WT or various mutants bind DNA *lacking* an AP-site, because there can be no cleavage product without an AP-site. AP endo is processive (5) as is uracil DNA glycosylase (3) and a number of restriction endonucleases (37,45). Being processive, the enzyme binds ds DNA randomly and then searches for an AP-site. After cleavage, the enzyme can dissociate or remain associated until it locates another lesion. However, experiments using EMSA allow us to examine binding to DNA lacking an AP-site. In all cases the enzyme binds DNA with an AP-site better than normal DNA. WT and most of the tyrosine mutant enzymes bind undamaged DNA with equal affinity. The exception is Y171F which binds undamaged DNA more strongly than does the WT. Because the enzyme is processive, we expected that Scatchard plot analysis of EMSA data should resolve into two components, one a high affinity binding site related to the presence of an AP-site and the other a lower affinity site related to non-specific binding to ds DNA (16,20). Unfortunately, when we examined the data by Scatchard analysis, we observed a decreasing convex function, indicating that at high enzyme concentrations there are multiple equilibria that are not independent of each other (16). This observation is consistent with previous reports by Barzilay et al. (1).

The fact that the presence of the hydroxyl group on Tyr<sup>171</sup> interferes with binding of AP endo to DNA lacking an AP-site is yet another indicator of fine tuning in distinguishing AP-site-containing DNA from undamaged DNA. In the co-crystal of AP endo with an 11-bp or a 15-bp AP-site containing oligomer (33) Tyr<sup>171</sup> does not interact directly with the AP-site. However, the rotation of the phenolate would enable a direct attack on the scissile phosphate without employing a water molecule as intermediate (35). The very same tyrosyl hydroxyl *diminishes* binding to DNA lacking an AP-site, perhaps because non AP-site DNA is less deformable than AP-site containing DNA. Thus, the tyrosyl group promotes discrimination between normal and AP-site containing DNA.

Not all proteins that interact with DNA can be studied by ST analysis. For proteins with enzymatic activity, there are two requirements: (1) the binding step must be completely dissociated from the enzymatic step and (2) once catalysis is initiated the enzyme must undergo one and only one round of catalysis. On the other hand, for proteins that bind DNA without chemically altering it or that turn over very slowly, such as transcription factors without enzymatic activity *per se* or glycosylases with a very low turnover number, one must use some

sort of physical strategy, of which EMSA is perhaps the simplest. Indeed, EMSA has proved a valuable tool for identifying consensus sequences for a variety of transcription factors (15, 18,39,46). Thus, the two methods together provide a more complete picture of how an active site tyrosine enables AP endo to bind and differentiate normal and AP-site containing DNA.

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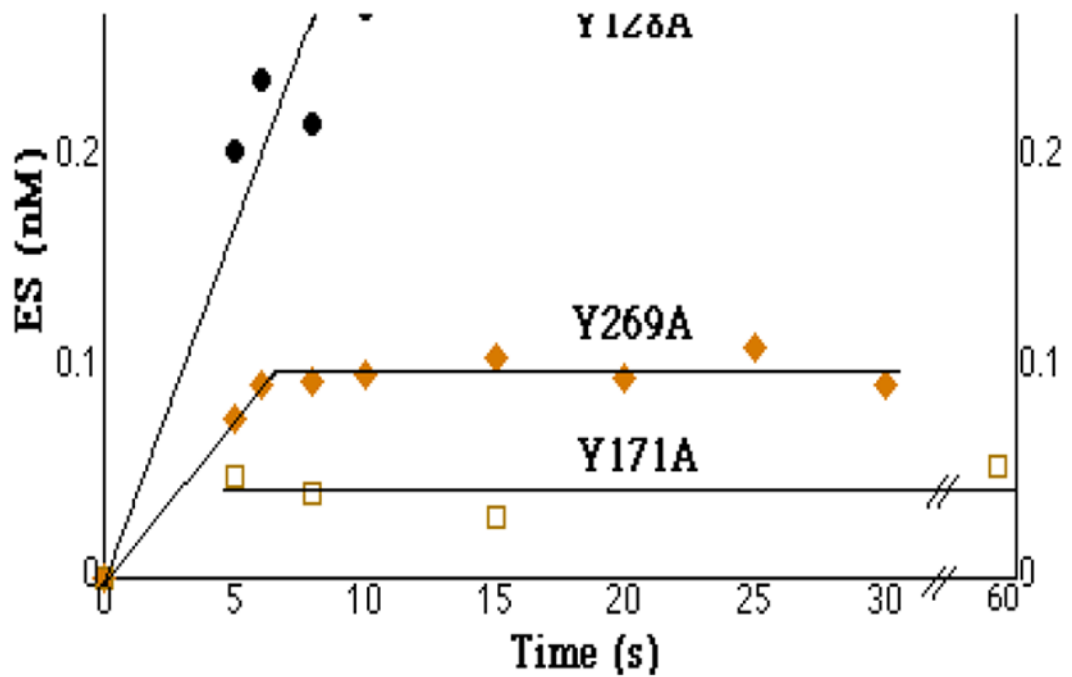
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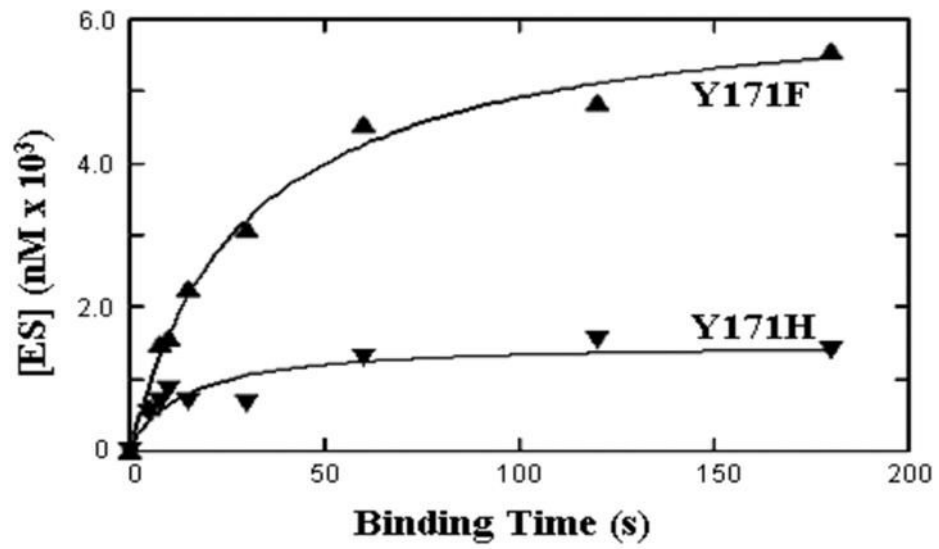
## Abbreviations

<b>AP endo</b>	Apurinic/aprimidinic endonuclease
<b>AP-site</b>	Abasic site
<b>ASR</b>	Abasic site repair
<b>dRP</b>	Phosphodeoxyribose
<b>ds</b>	Double stranded
<b>EDTA</b>	Ethylene diamine tetraacetic acid
<b>EMSA</b>	Electromobility shift assay
<b>ES</b>	Enzyme.substrate complex

<b>HDP</b>	Heat degradation product
<b>HEPES</b>	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
<b>SS</b>	Steady state
<b>ST</b>	Single turnover
<b>UNG</b>	Uracil DNA glycosylase ( <i>E.coli</i> )
<b>WT</b>	Wild type

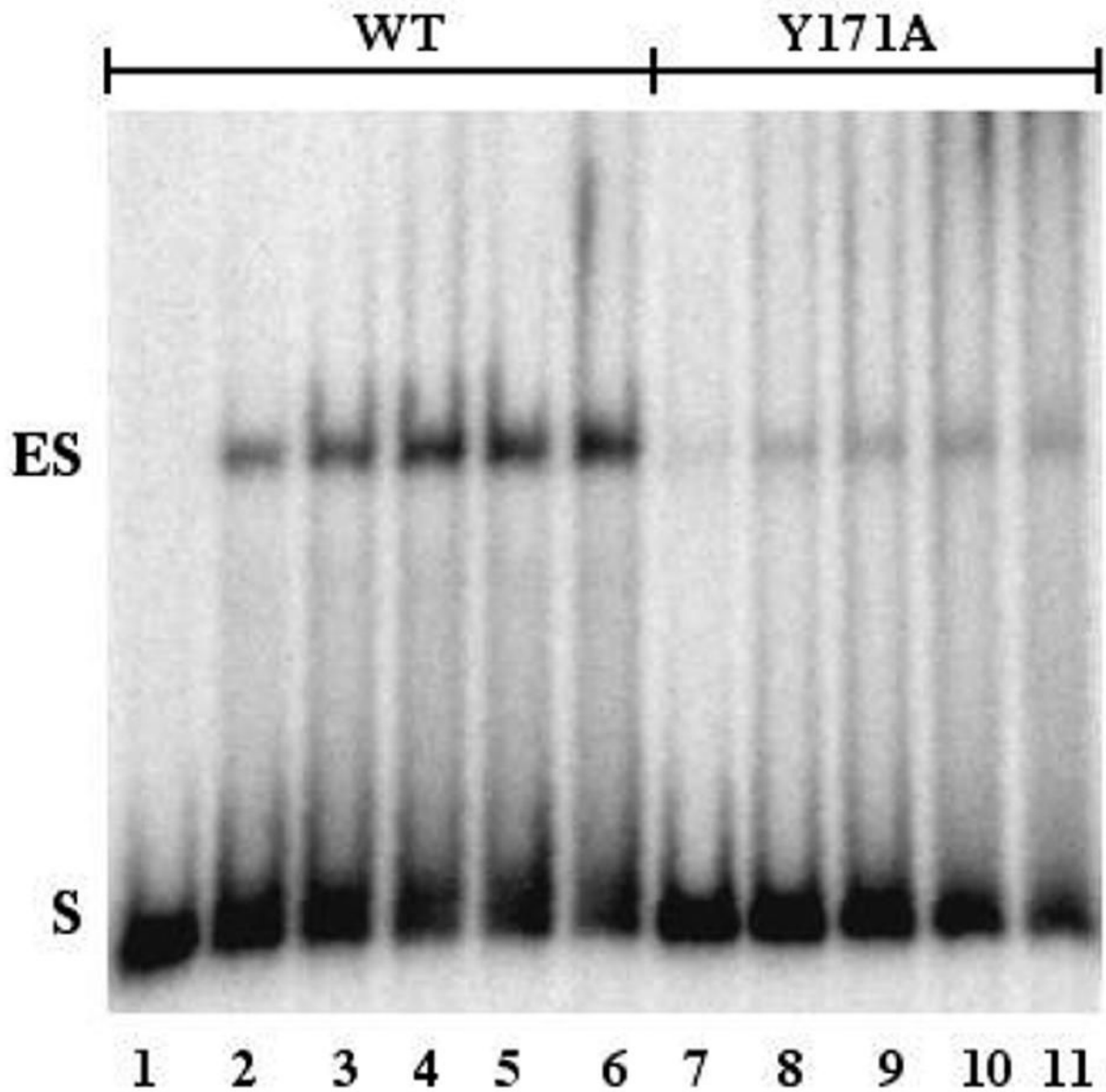


**Figure 1.** Single turnover binding of AP-site containing DNA by AP endonuclease mutants Y128A (●), Y269A (◆) and Y171A (□). For the experiment shown in the figure, substrate and enzyme were each present at 4 nM for Y128A, at 4 nM and 0.4 nM respectively for Y269A and at 7 nM for Y171A.  $[ES]_{eq}$  for Y128A was 0.28 nM, when initial concentration of enzyme and substrate were each 4 nM. ( $[ES]_{eq}$  for Y269A was 0.1 nM when initial enzyme and substrate were 0.4 nM and 4 nM respectively).



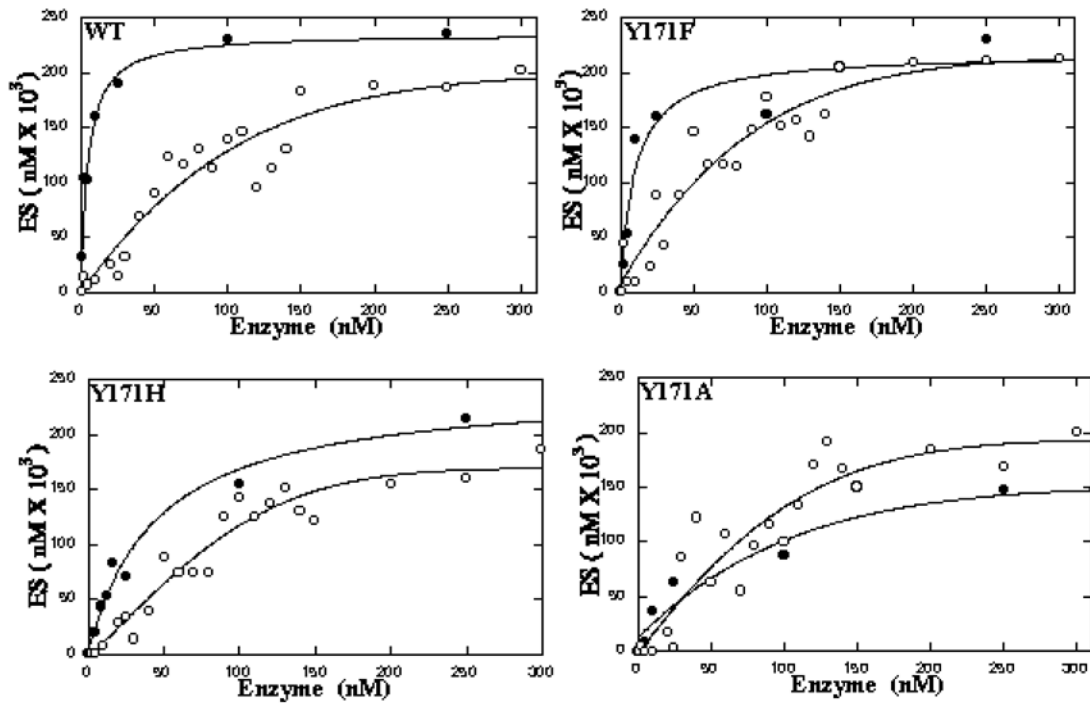
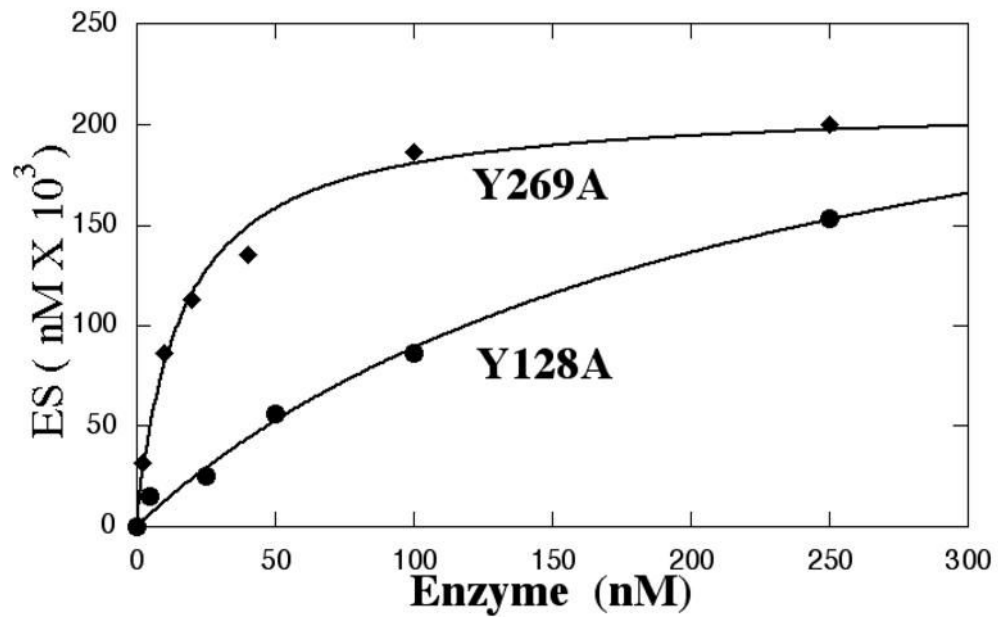
**Figure 2.** Single turnover binding of AP-site-containing DNA by AP endo mutants Y171F (▲) and Y171H (▼). For the experiment shown in the figure, substrate and enzyme were each present at 7 nM (Y171F) or 4 nM and 20 nM respectively (Y171H).





**Figure 3.**

EMSA analysis of binding AP-site containing DNA by wild type and Y171A AP endonuclease, non-denaturing gel. WT or Y171A were incubated with 0.25 nM substrate for 30 min at 25 ° C and resolved by non-denaturing gel electrophoresis as described in Methods. Lane 1, no added protein; lane 2, 0.5 nM WT; lane 3, 2.5 nM WT; lane 4, 5.0 nM WT; lane 5, 10 nM WT; lane 6, 25 nM WT; lane 7, 5 nM Y171A; lane 8, 25 nM Y171A; lane 9, 50 nM Y171A; lane 10, 100 nM Y171A; lane 11, 250 nM Y171A.



**Figure 4.**

Comparison of binding AP-site containing DNA with (●, ◆) and without (○) an AP-site by EMSA analysis. WT and mutant proteins at the indicated concentrations were incubated with 0.25 nM substrate and resolved by non-denaturing gel electrophoresis as described in Methods. The distribution of bound and unbound substrate was quantitated by phosphorImager analysis. A: Binding AP-site-containing DNA by Y128A(●) and Y269A (◆) B: Binding by WT AP endo or Tyr<sup>171</sup> mutants to oligonucleotide with (●) or without(○) an abasic site.

**Table 1**

Binding constants obtained by steady state and single turnover methodologies. Single turnover data are from experiments presented here;  $K_m$  and enzymatic efficiency data are from Mundle et al.(35).  $K_m$  for an enzyme such as AP endo that follows Briggs-Haldane kinetics (2) is described by the equation  $K_m = (k_{cat} + k_{-1})/k_{+1}$ . When  $k_{cat} < k_{-1}$ ,  $K_d$  is best approximated by the  $K_m$  obtained by steady state measurements. In these cases  $[ES]_{eq}$  measured by single turnover is an *underestimate*, which results in an *overestimate* of  $K_d$ . Hence, the  $K_m$  determined by steady state kinetics is the better estimator of  $K_d$ . (See text.)

Mutant	Abasic Site	$K_m$ (nM) <sup>(1)</sup>	$k_{cat}$ (s <sup>-1</sup> ) <sup>(1)</sup>	Enzyme Efficiency <sup>(1)</sup> (M <sup>-1</sup> s <sup>-1</sup> )	$K_d$ <sup>(2)</sup> (nM)
Wild Type	+	100	10	$1 \times 10^8$	0.8 <sup>(1)</sup>
Y269A	+	165	1.3	$8 \times 10^6$	7±0.5
Y128A	+	140	0.5	$3 \times 10^6$	52±22
Y171F	+	255	$9 \times 10^{-4}$	$4 \times 10^3$	255
Y171H	+	>2000	$4 \times 10^{-3}$	$2 \times 10^3$	>2000
Y171A	+	360	$8 \times 10^{-4}$	$2 \times 10^3$	360

<sup>(1)</sup>Data from Mundle et al.(35).

<sup>(2)</sup> $K_d$  values derived from single turnover (3 or 4 independent experiments for each mutant) or steady state experiments<sup>(1)</sup>, as appropriate (see text).

**Table 2**

Ability of WT and tyrosine mutants to discriminate DNA with and without an abasic site.  $K_d$  values  $\pm$  SD for the tyrosine mutants were calculated from binding by EMSA analysis as described in the text and shown in Figure 4. The ratio of the  $K_d$  value for DNA containing an AP-site to that for DNA without an AP-site is the discrimination index. ND, not done

Mutant	$K_d^{(1)}$ (EMSA) (nM)	$K_d^{(2)}$ (EMSA) (nM)	Discrimination Index (EMSA) <sup>(3)</sup>
	With abasic site	Without abasic site	
Wild Type	4 $\pm$ 0.7	67 $\pm$ 9	17
Y269A	23 $\pm$ 11	ND	–
Y128A	63 $\pm$ 15	ND	–
Y171F	8 $\pm$ 1	43 $\pm$ 2	5
Y171H	34 $\pm$ 13	64 $\pm$ 7	2
Y171A	70 $\pm$ 18	65 $\pm$ 9	1

<sup>(1)</sup> These values are the average of 4–8 independent experiments for each mutant or WT enzyme. The values in parenthesis are standard errors of the mean.

<sup>(2)</sup> These values are the average of 6–9 independent experiments. The values in parenthesis are standard errors of the mean. ND, not done

<sup>(3)</sup> Ratio of  $K_d$  for DNA lacking an AP-site to  $K_d$  for DNA with an abasic site