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Impact of abasic site orientation within nucleosomes on human APE1 endonuclease activity

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Abstract

Glycosylases responsible for recognizing DNA lesions and initiating Base Excision Repair (BER) are impeded by the presence of histones, which are essential for compaction of the genetic material in the nucleus. Abasic sites are an abundant mutagenic lesion in the DNA, arising spontaneously and as the product of glycosylase activity, making it a common intermediate in BER. The apurinic/apyrimidinic endonuclease 1 (APE1) recognizes abasic sites and cleaves the DNA backbone adjacent to the lesion, creating the single-strand break essential for the subsequent steps of BER. In this study the endonuclease activity of human APE1 was measured on reconstituted nucleosome core particles (NCPs) with DNA containing enzymatically-created abasic sites (AP) or the abasic site analog tetrahydrofuran (TF) at different rotational positions relative to the histone core surface. The presence of histones on the DNA reduced APE1 activity overall, and the magnitude was greatly influenced by differences in orientation of the lesions along the DNA gyre relative to the histone core. Abasic moieties oriented with their phosphate backbones adjacent to the underlying histones (In) were cleaved less efficiently than those oriented away from the histone core (Out) or between the In and Out orientations (Mid). The impact on APE1 at each orientation was very similar between the AP and TF lesions, highlighting the dependability of the TF abasic analog in APE1 activity measurements in nucleosomes. Measurement of APE1 binding to the NCP substrates reveals a substantial reduction in its interaction with nucleosomes compared to naked DNA, also in a lesion orientation-dependent manner, reinforcing the concept that reduction in APE1 activity on nucleosomes is due to occlusion from its abasic DNA substrate by the histones. These results suggest that APE1 activity in nucleosomes, like BER glycosylases, is primarily regulated by its chance interactions with transiently exposed lesions.

Keywords

nucleosome; histone; DNA damage; APE1 endonuclease; tetrahydrofuran

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Conflict of interest statement

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1. Introduction

Though DNA is a robust and stable molecule, making it useful as the genetic material in most organisms, its composite nucleotides are amenable to chemical modifications that can corrupt and change the information it encodes. Human cells acquire tens of thousands of spontaneous chemical modifications to the DNA, and the repair process of base excision repair (BER) is responsible for removal of the most abundant lesions, including base oxidation and alkylation [1, 2]. BER is a coordinated, stepwise process primarily initiated by recognition and removal of a modified nucleobase by a DNA glycosylase, creating an abasic site [2, 3]. Abasic sites (or AP site, for apurinic/apyrimidinic) are mutagenic and potentially cytotoxic BER intermediates that can also form spontaneously from hydrolysis of the N-glycosidic bond in nucleotides [4, 5]. The BER protein APE1 is the primary AP endonuclease in human cells responsible for recognition of AP sites in the DNA [6]. APE1 cleaves the sugar-phosphate backbone on the 5' side of the abasic deoxyribose phosphate residue [7], providing the nicked DNA substrate for DNA polymerase β (Pol β). This repair polymerase removes the 5' abasic deoxyribose phosphate moiety and performs the DNA synthesis step of BER, directed by the intact complementary strand. The repair process is completed by subsequent ligation of the nicked strand to restore continuity of the repaired strand [5].

APE1 recognition of abasic sites is essential for cell survival, as AP sites greatly impede DNA replication [8, 9]. Indeed, in mouse cells defects in APE1 are embryonic lethal and elimination of APE1 expression causes apoptosis [10]. Importantly, APE1, like the other steps of BER, must function efficiently in the context of chromatin.

The compaction of DNA into chromatin in cells is essential for its containment to the nucleus and regulation of important metabolic events, including transcription and replication. The primary level of compaction consists of the nucleosome core particle (NCP), entailing 147 pb of DNA wrapped ~ 1.7 times around an octamer of histone proteins. The octamer consists of two each of four different histone proteins (H2A, H2B, H3, and H4), which makes numerous contacts with the associated DNA and causes localized distortions to the DNA helix. The nucleosome impedes binding factors from its associated DNA by directly occluding them at intervals along the helix where the DNA is in direct contact with the histones, and more generally by reduction of DNA mobility and maintenance of local structural features that prohibit protein-DNA interactions [11, 12].

Many studies have focused on determining the impact of histones on the initiation of BER, measuring activity of glycosylases on their associated lesion substrates in nucleosomes [13–21]. Covering a range of glycosylases, all of these studies conclude that histones do indeed interfere with the ability of a glycosylase to act upon its target substrate. In general, the orientation of the lesions on the DNA impacts the accessibility of the glycosylase to its substrate, such that those nucleotides with their phosphate backbones against the surface of the histone octamer (generally referred to as inwardly-oriented), are converted to product less efficiently than those in an outward orientation. Not surprisingly the quantitative differences in activity on the In and Out oriented lesions vary among the studies due to

experimental variations including strength of the nucleosomal positioning sequence, lesion type, and glycosylase used, but the overall conclusions are consistent.

Importantly, there remains a lack of information regarding the impact of histones on the step of BER after glycosylase-directed AP site formation, the detection and cleavage of the intermediate by APE1. Histone-associated inhibition of APE1 activity on NCPs seems probable, as its mode of DNA binding and mechanism of substrate extraction from the DNA duplex is similar to that of DNA glycosylases [22, 23]. In addition, for APE1 to perform its nucleolytic activity there is dependence on structure specificity of the substrate, requiring the DNA to be bent by 35° [23].

The abasic site analog tetrahydrofuran (TF) has been used in most studies as a substitute for abasic sites since it was first employed [24], as it can be easily incorporated into oligonucleotide substrates as a nucleoside analog and is thermally and chemically more stable than the biologically-relevant, glycosylase generated 2'-deoxyribose AP sites (See figure 1A for structures of the two abasic sites) [25]. The TF is a stable ring structure, whereas the AP site is actually an equilibrium mixture of tautomers consisting primarily of α and β anomers of deoxyribofuranose and a low level of the partially hydrated (open chain) form (which themselves are readily subject to a β -elimination reaction leading to single strand breaks in the DNA) [25]. Thus, in solution, the two abasic lesions are dynamically different, and it is unclear whether the TF analog acts as a relevant substitute in conditions where the bending of the DNA (due to association with the histones) plays a key role in interaction with its enzyme.

In this study the endonucleolytic activity of APE1 is measured on abasic sites within the context of nucleosomes at various rotational orientations in the DNA helix with respect to the histones. Two nucleosomal positioning sequences of different strengths are used, including the strongly positioned model 601 sequence and the TG positioning motif. The lesion sites chosen for this study in the TG positioning sequence, representing three different helical orientations relative to the histones (In, Mid, and Out), are identical to those used previously to measure glycolytic activity of human uracil DNA glycosylase (UDG) in nucleosomes with uracil residues at those locations [18]. This allows direct comparison between APE1 activity and the initiating step of BER at three distinct, well-defined lesion orientations. In addition, APE1 endonuclease activity is measured for both the natural AP site substrate and the TF abasic analog at each nucleosome orientation, providing the important comparison between these two distinct model lesions. Finally, the effect of histones and lesion orientation on binding of APE1 to NCPs is assessed to determine if affinity for the abasic site is consistent with reduction in APE1 endonuclease activity in nucleosomes.

2. Materials and methods

2.1 NCP Substrate Preparation

The double-strand DNA substrates used in this study entailed one of two distinct nucleosomal positioning sequences, the 150 bp TG motif or the 147 bp 601 sequence, both created by the annealing of full length complementary oligonucleotides (synthesized by

solid phase synthesis by Midland Certified Reagent Company, Midland, TX). The TG substrate contained a central 15 bp GRE sequence flanked by the TG nucleosomal positioning sequences [11, 18, 26]. For TF DNAs, three oligos (TF-IN, TF-MID, TF-OUT) each contained a tetrahydrofuran abasic analog at a different position within the GRE and one oligo (PARTNER) served as the partner strand for annealing with the rest. Three additional oligos were also used to create the AP site DNA (U-IN, U-MID, U-OUT), which contained uracil residues that were subsequently converted to abasic (AP) sites (below). Sequences of the GRE at the center of each of the three oligos are as follows, where X marks the TF or uracil residue: GRE: U-In: TGTACAGCATGTTXT; U-Mid: TGTAXAGCATGTTCT; U-Out: TGTACAGXATGTTCT. See figure 1B for location relative to the dyad axis on the nucleosome. A slightly modified version of the 601 DNA sequence [27] was used to place uracil residues or TF analogs at distinct positions near the dyad axis, as has been used previously and demonstrated to have robust and consistent positioning [28], the locations of which are shown in figure 1B. Prior to annealing with the partner strand, oligos were radiolabeled with ^{32}P at the 5' ends with T4 polynucleotide kinase (Invitrogen) and $\gamma\text{-}^{32}\text{P}\text{-ATP}$ (PerkinElmer) for 30 minutes at 37° C. Creation of AP DNAs was done by treatment of the uracil DNAs with recombinant *E. coli* UDG (New England Biolabs) for 30 minutes (reaction buffer: 25 mM HEPES (pH 7.5), 2 mM DTT, 0.2 mM EDTA, 100 $\mu\text{g/ml}$ BSA, 10% glycerol, 5 mM MgCl_2 , 4 mM ATP), the reactions terminated with addition phenol:chloroform:isopropanol (PCI; 20:19:1). TF and AP site molecular comparisons are shown in figure 1A.

Mononucleosomes were prepared by histone octamer transfer, combining the radiolabeled 150 bp DNA substrates with chicken erythrocyte core particles prepared from chicken erythrocytes [29] at high ionic strength, and subsequent incremental dialysis as described previously [30, 31] (Fig. 1C, TG motif; Fig. 1D, 601 sequence). Briefly, 6 pMol of DNA was mixed with 300 pMol erythrocyte NCPs (a 1:50 ratio of labeled DNA to chicken NCP) in 100 μl TE buffer at 4 M NaCl. The mixture was transferred to 3500 mW dialysis units (Thermo Scientific), which were placed for 1 hour in 500 ml of TE at 600 mM NaCl and subsequently for 1 hour in 500 ml of TE at 50 mM NaCl. The final stock concentration of labeled NCPs is 60 nM, with the total NCP concentration, including the unlabeled NCPs, at 3 μM .

2.2 APE1 endonuclease activity measurements

Treatment of DNAs and NCPs with recombinant, full-length human APE1 (kindly provided by Sam Wilson, NIEHS) were initiated by adding APE1 to a final concentration of 5 nM in 20 μl reactions in the time-dependent activity assay. 0.1 pMol of naked DNA or 6 pMol of NCPs were used in 20 μl endonuclease reactions (for final concentrations of 5 nM and 300 nM, respectively), and incubations were at 37°C for 5, 10, 15, 30 and 60 minutes, in the same buffer as the UDG-treatments of the U DNAs (above in 2.1) and as used previously [18]. In the APE1 concentration-dependent activity assays, reaction volumes and NCP substrate concentrations were maintained as above, but substrates were incubated in variable concentrations of APE1 (.1 nM to 20 nM) for 60 minutes. Reactions were terminated with addition phenol:chloroform:isopropanol (PCI; 20:19:1).

All naked DNA samples had chicken erythrocyte core particles added to a concentration of 300 nM to adjust for the excess core particles present in reconstituted nucleosome samples. All samples were boiled and separated on 10% polyacrylamide (0.5% bisacrylamide) 7M Urea denaturing gels, to resolve APE1 cleavage at abasic sites. DNAs with AP lesions were treated with 1M sodium borohydride (NaBH₄) for 20 minutes on ice immediately after APE1 reaction termination to reduce remaining uncleaved AP residues and prevent their breaking during sample boiling and electrophoresis. Gels were run in 1X TBE buffer, exposed to PhosphorImager screens (Molecular Dynamics), visualized on a STORM 840 PhosphorImager (Amersham), and images analyzed with IMAGEQUANT software (Molecular Dynamics).

2.3 AP-DNA binding assay

Different concentrations of APE1 protein (0 – 1.6 μ M) were incubated in 20 μ l reactions with 6 pMol (300 nM) associated AP-In and AP-Out NCP substrates for 10 min on ice in the enzymatic reaction buffer used in the endonuclease activity assays in section 2.1 (but without ATP or MgCl₂). APE1 incubations were also performed on 6 pMol (300 nM) of uracil-containing U-Out NCPs, and on naked AP-In DNA (.12 pMol, 60 nM; in the presence of additional chicken NCPs at a concentration of 300 nM), at an APE1 concentration range 0 – 50 nM). DNA/NCP binding was resolved on a non-denaturing 6% polyacrylamide gel. Substrates were visualized and quantified as done in section 2.2.

3. Results and discussion

3.1 Activity of APE1 on AP sites and TF moieties on naked DNA shows no sequence bias for sites tested

Prior to measuring APE1 endonuclease activity on abasic sites in nucleosomes, cleavage activity by APE1 was determined at each site on naked DNAs to test for possible sequence-dependent cleavage bias. DNAs with the abasic sites (either AP or TF) at residues associated with the In, Mid and Out orientations of the TG sequence DNAs and the In and Out orientations of the 601 sequence DNAs were treated with a low concentration of APE1 (0.1 nM) over the course of 1 hour, products were separated on denaturing gels (Fig. 2A) and the percentage of cleaved product was determined at different incubation times (Fig. 2B and C). The reactions were done in the presence of unlabeled chicken NCPs (at 300 nM) to match the background levels of excess NCPs in subsequent nucleosome experiments. Though there was a clear difference between the two abasic substrates (TF or AP), the DNAs of each type of lesion showed very similar rates of product formation, independent of their sequence context. These results are consistent with previous observations of APE1 endonuclease activity, which generally shows little, if any, sequence bias [32]. The incision product maxima (dashed lines in Fig. 2B and C) were also determined for each lesion set (85% for the AP lesions in both the 601 and TG sequences, 80% for the TF lesions in the 601 DNAs, and 67% for the TF lesions in the TG sequence DNAs), which represent the actual fraction of abasic substrate available for cleavage (since some fraction of the DNA oligos lack the TF and AP sites due to errors in synthesis and, in the case of the AP sites, lack of complete conversion from uracil residues). These values were used in subsequent APE1 endonuclease

activity measurements to correct for the lack of complete substrate availability in the samples.

3.2 Endonuclease activity of APE1 on abasic sites in nucleosomes is highly impacted by presence of histones and lesion orientation

To measure the endonuclease activity of APE1 on nucleosomes, NCP substrates with AP and TF abasic sites were incubated with 5nM APE1 concentrations over the course of one hour and corrected for the fraction of available substrate, as determined in section 3.1 (Fig. 3A and B). The activity of APE1 was greatly impacted by rotational orientation of the lesion, with clear differences between among the In, Mid and Out orientations in the NCPs with the TG positioning sequence and the In and Out orientations in the NCPs with the 601 positioning sequence. The Out orientations of both lesion types for each NCP was also measured 0.1 nM and the 60 minute time point shown. This concentration allowed for better detection of any potential differences between the AP site and TF analogs at the Out orientation, which were quickly cleaved at the 5 nM APE1 concentration, and allowed for comparison with the APE1 concentration used on the naked DNA measurements). These results are consistent with a previous study that measured the APE1 phosphodiesterase activity for removal of 3'-phospho- α , β -unsaturated aldehyde groups (product of the bifunctional glycosylase hNTH1) at two different orientations in nucleosomes [20]. Though the difference in APE1 activity between the In and Out orientations in that study was not as large as those seen for this study, due in part to experimental differences including nucleosomal positioning sequence and substrate (nicked DNA vs. abasic site), both studies support the conclusion that activity of APE1 at lesions in nucleosomes is based primarily on its ability to interact with the lesion.

The increased difference between the In and Out orientations in the 601 NCPs compared to those two orientations in the TG NCPs (compare figures 3A and 3B) is not too surprising, considering the increased positioning strength of the 601 sequence, likely due to greater restriction of movement of the DNA on the histone octamer relative to that of the TG NCP [33, 34]. Importantly, there appeared to be little difference in APE1 activity between the glycosylase-generated AP sites and the TF analogs at any orientation on either nucleosome substrate, with the reduction of activity associated with the inwardly oriented lesions being nearly identical for both lesion types within each of the two differently-positioned NCPs (Fig. 3). In order to test the possibility that detection of differences between the two lesions may be more sensitive to APE1 concentration rather than reaction time, endonuclease product formation was also assessed for 60 minutes over a wide range of APE1 concentrations. Figure 4 shows the concentration-dependent APE1 activity on NCPs with AP sites and TF analogs at the In and Out orientations in the 601 positioning sequence. Consistent with the time-dependent results, there was a large difference in product formation between the lesion orientations, with the Outward-oriented lesions showing formation of the cleavage product at the lowest APE1 concentration tested (0.1 nM, ~15% product). The inwardly-oriented lesions did not show detectable cleavage products until the concentration of APE1 reached 1 nM (~12% product). Also consistent with the APE1 activity time course, there was little difference between the AP site and TF analog lesions in cleavage by APE1. These results support the value of using the stable TF analog in nucleosome studies and

highlight that the structural differences between the two lesions do not provide a major difference in the accessibility of APE1 in histone-associated DNAs.

The APE1 endonuclease results on the NCPs with the TG positioning sequence reveal additional facets of BER in nucleosomes when compared to the human UDG activity measured in our previous study using the same NCP substrates with uracil residues at each orientation [18]. The differences in enzyme activity between the In and Out orientations were greater for UDG (which showed a larger difference in product formation between the In and Out, despite the greater increase in enzyme concentration for the In orientation to get detectable activity). This may be in part associated with differences in the lesions, as AP sites cause minor local distortions in the helix [35], distinct from the non-helix distorting uracil residue, possibly favoring an increase in APE1 activity on inward-oriented lesions where increased helical distortion may make such lesions more accessible. However, another key distinction between the two studies is the minimal impact the histones had on UDG activity for the outward oriented lesion. APE1 activity is greatly reduced in nucleosomes compared to naked DNA, implying that the endonuclease-substrate interaction is sensitive to the proximity of histones even without direct occlusion of the lesion. This difference between UDG and APE1 may be associated with a stricter requirement for DNA flexibility by APE1 for DNA binding and/or enzymatic activity, or may reflect a fundamental disruption of APE1 activity in the chemical microenvironment of the nucleosome.

3.3 APE1 binding to nucleosomes is impacted by lesion orientation

Presumably the reduction in APE1 activity on nucleosomes is the result of reduced accessibility to lesions due to the presence of histones associated with the DNA. To test that assumption, nucleosome substrates with AP sites at the In and Out orientations (AP-In and AP-Out) were incubated with a range of APE1 concentrations and assessed for shifts in electrophoretic mobility on non-denaturing polyacrylamide gels (Fig. 5A). Binding of the nucleosomes by APE1, as evidenced by formation of a band with reduced mobility, was apparent for both nucleosome substrates, but was detectable in the AP-Out at a lower concentration than that seen for AP-In. The concentration range necessary to detect binding to nucleosomes was much higher than that required for binding to the naked DNA substrate (AP-In DNA; Fig. 5B), which shows binding to APE1 greater than 10-fold reduction in concentration. The binding to the AP substrates is lesion-specific, as there was no binding detected to a nucleosome substrate containing a uracil residue in the Out orientation (U-Out) within the APE1 concentration range shown (Fig. 5B). Quantification for comparison between the APE1 binding of the two nucleosomes tested is shown in figure 5C, highlighting the lesion-orientation associated differences in APE1 binding to the substrates. Thus, the reduction of APE1 endonuclease activity on nucleosomes appears to be primarily influenced by physical interaction with its substrate, consistent with the disparity between naked DNA and nucleosomes as well as the impact of lesion orientation.

3.4 Concluding remarks

APE1 has reduced endonuclease activity on abasic sites in the context of histones, related to a reduced ability to bind its substrate lesions. It seems probable that, in cell nuclei, other

factors assist APE1 (and perhaps all of the BER proteins), in overcoming the chromatin-associated obstacles to their activity. Though it is likely that BER is initiated by binding of a repair protein with its lesion [36], there remains the conundrum of how other factors could assist this molecular recognition without specific detection of the lesion prior to repair. Though the answer to this question remains elusive, it is clear that the APE1 endonuclease step of BER is greatly impacted by occlusion of its substrate in the first order of chromatin compaction.

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Highlights

- APE1 endonuclease activity is reduced on nucleosomes
- Orientation of abasic site relative to histone octamer determines extent of reduction
- Reduced APE1 activity correlates with lower binding affinity to nucleosomes

Figure 1. Relative positions of AP and TF sites and NCP reconstitution verification

A. Molecular depictions of the two abasic sites used in the study, the biologically relevant apurinic/aprimidinic (AP) site and the tetrahydrofuran (TF) abasic site analog. **B.** GRE sequence (5' to 3') near NCP dyad in the TG positioning sequence and the 601 positioning sequence near its dyad with location of cytidine residues replaced by AP sites or TF residues within DNA substrates: AP/TF-In, -Mid (in the TG positioning sequence only), and -Out. Examples of non-denaturing polyacrylamide gels showing slowed migration of DNA substrates after reconstitution into nucleosome core particles of the TG-positioned NCPs (**C**) and 601-positioned NCPs (**D**).

Figure 2. Relative rates of APE1-directed cleavage of naked DNA substrates

A. Denaturing polyacrylamide gels showing radio-labeled full length (FL) substrate and cleavage product (CP) of AP and TF DNAs substrates with the TG and 601 positioning sequences. For charts, cleavage of Inward oriented lesions (Circles), Mid oriented lesions (Triangles), and Outward oriented lesions (Squares) are measured for both AP sites (closed shapes) and TF analogs (open shapes). **B.** Cleavage of naked DNA substrates as a function of time with .1 nM APE1 of the TG positioning sequence; **C.** Cleavage of naked DNA substrates with the 601 positioning sequence. Solid lines are fit to the data. Dashed lines near the top of each chart represent maximum percentage of product formed for each group of substrates, indicating actual total substrate availability. Each data point represents the mean of at least 3 independent experiments and error bars represent standard deviation.

Figure 3. Relative rates of APE1 activity on AP site and TF analog NCP substrates

NCP constructs were incubated with 5 nM APE1 for 0 – 60 minutes and percent cleavage product was determined for NCPs with differently oriented lesions in the TG positioned NCPs (**A**) and 601 positioned NCPs (**B**). AP sites are represented as solid symbols; TF analogs represented as open symbols. Lesion orientations are defined by their designated symbol: In (Circles), Mid (Triangles), and Out (Squares). A single line is used to represent both lesion types at each orientation. Note: A 60 minute time point of the 0.1 nM APE1 incubation for the TF/AP-Out data are also shown, shifted to the right of 60 minutes for graph clarity. Each data point represents the mean of at least 3 independent experiments and error bars represent standard deviation.

Figure 4. Cleavage of NCPs as a function of APE1 concentration

NCP constructs were incubated with variable concentrations of APE1 (0 – 20 nM) for 60 minutes and percent cleavage product was determined for 601 sequence-positioned NCPs with differently oriented lesions. **(A)** Denaturing polyacrylamide gels showing radio-labeled the full length (FL) substrate and cleavage product (CP) of AP-containing NCP substrates. **(B)** Percent cleavage of each NCP substrate as a function of APE1 concentration, with inward oriented (Circles) and outward oriented (squares) AP sites (Closed shapes) and TF analogs (Open shapes). Each data point represents the mean of at least 3 independent experiments and error bars represent standard deviation.

Figure 5. Binding of APE1 to AP-NCPs

A. Increasing amounts of APE1 were added to TG AP-In and AP-Out nucleosome substrates and assessed on non-denaturing 6% polyacrylamide gels for assessment of APE1 binding affinity for the AP-In and AP-Out orientations. **B.** Binding controls for APE1: Naked DNA with a single AP site (corresponding to the In orientation) and NCPs with a uracil residue in the Out orientation incubated with increasing amounts of APE1. (Note: the APE1 concentrations for the naked DNA are in nM, whereas the concentrations for the U-Out NCPs are in μM) **C.** Plot of A, percentage of NCPs in complex for each concentration of APE1 (AP-Out represented as black bars, AP-In represented as white bars). Values represent the mean of three different experiments and the error bars represent standard deviations.