Evidence for a Common Active Site for Cleavage of an AP Site and the Benzene-Derived Exocyclic Adduct, 3,N^4-Benzetheno-dC, in the Major Human AP Endonuclease†

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ABSTRACT: We have previously reported that the 3,N^4-benzetheno-dC (*p*-BQ-dC) endonuclease activity found in HeLa cells is a novel function of the major human AP endonuclease (HAP1) [Hang *et al*. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 13737−13741]. In this study, we compare the enzymatic and biochemical properties of the enzyme toward *p*-BQ-dC and an AP site in a defined oligonucleotide. A comparative analysis of the specificity constants ($K_{cat.}/K_m$) for *p*-BQ-dC and an AP site indicates that the AP site is the preferred substrate. The enzyme does not cleave other structurally related exocyclic adducts and modified nucleosides such as 1,N^6-etheno-dA, 3,N^4-etheno-dC, 1,N^2-etheno-dG, 1,N^2-propano-dG, 8-oxo-dG, and thymine glycol. The *p*-BQ-dC activity requires a double-stranded DNA substrate and is affected by the base in the opposite strand, with maximal activity for a *p*-BQ-dC·G pair and minimal activity for a *p*-BQ-dC·C pair. The *p*-BQ-dC activity also requires Mg^{2+}, Mn^{2+}, or Zn^{2+} with optimal concentration spectra similar to those for the AP function. The optimal pH ranges for these two functions are also similar to each other (5.5−6.5). Six mutant HAP1 proteins containing single amino acid substitutions were assayed in parallel for comparison of their activities toward *p*-BQ-dC and the AP site. These mutants either concomitantly lost (N212A, D210N) or had reduced (D219A, E96A, and N212Q) or unchanged (H116N) *p*-BQ-dC and AP activities. This parallelism strongly supports the hypothesis that cleavage of *p*-BQ-dC requires the same catalytic active site as that proposed for the AP function. This dual activity toward two structurally unrelated substrates, an AP site and a bulky exocyclic adduct, has implications for substrate recognition. The AP site and *p*-BQ-dC cause different changes in the local conformation around the lesion as it is visualized by molecular modeling.

Benzene has been recognized for its harmful effects for nearly 100 years. Today, benzene is classified as a human carcinogen (IARC, 1987), based on the high levels of leukemia in workers exposed to this chemical. Benzene is widely used, and it is the chemical produced in the 15th largest quantity in the United States (Hricko, 1994). Although its metabolism *in vivo* is still unclear, one pathway (Huff *et al*., 1989) was found to lead to the stable metabolite, *p*-benzoquinone (*p*-BQ), which is the most potent mutagen of 12 structurally related simple benzoquinones tested in the Ames Salmonella assay (Hakura *et al*., 1995) and is also carcinogenic in rodents (Huff *et al*., 1989). *p*-BQ has been found to form cyclic benzetheno derivatives *in vitro* with the DNA bases dA (Pongracz & Bodell, 1991), dC (Pongracz *et al*., 1990), and dG (Jowa *et al*., 1986, 1990). However, studies on repair only became possible when 3,N^4-benzetheno-2′-dC, 1,N^6-benzetheno-2′-dA, and 1,N^2-benzetheno-2′-dG were synthesized, converted to the phosphoramidites, and site-specifically incorporated into defined oligonucleotides (Chenna & Singer, 1995, 1997).

We had previously reported that HeLa cells contained nicking activities toward the benzetheno derivatives in such defined oligomers containing *p*-BQ-dC or *p*-BQ-dA. On the basis of the cleavage pattern using cell-free extracts and partially purified protein, it was presumed that a DNA glycosylase was responsible (Chenna *et al*., 1995). Most recently, on the basis of sequence homology and substrate specificities using purified human *p*-BQ-dC endonuclease and recombinant AP endonuclease, we found that this *p*-BQ-dC nicking is actually carried out by the major human AP endonuclease (Hang *et al*., 1996b), variously called HAP1 (Robson & Hickson, 1991), Ape (Demple *et al*., 1991), or APEX (Seki *et al*., 1992)). This enzyme, as well as *E. coli* exonuclease III and endonuclease IV, directly incises the oligonucleotide 5′ to the *p*-BQ-dC adduct without prior generation of an AP site, resulting in the *p*-BQ-dC left as a "dangling base" on the 5′ terminus (Hang *et al*., 1996b).

The above three enzymes involved in the cleavage of the *p*-BQ-dC-containing oligonucleotide are all "class II" AP endonucleases, which hydrolyze the phosphodiester bond on the 5′ side of an AP site (Demple & Harrison, 1994; Barzilay & Hickson, 1995). The recent finding that the bulky exocyclic *p*-BQ-dC adduct is a substrate for these AP endonucleases implies that the substrate range of AP endonucleases may be much broader than hitherto imagined. A

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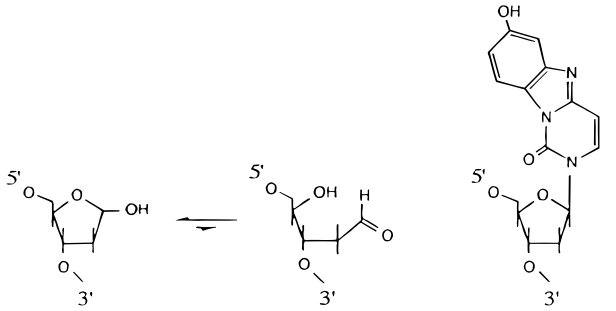


FIGURE 1: (Left) structures of two forms of an AP site formed by release of uracil using uracil−DNA glycosylase. The "regular" AP site is a mixture of the major ring-closed form and the minor ring-opened form. (Right) structure of 3,N^4-benzetheno-2′-deoxycytidine (*p*-BQ-dC).

detailed understanding of the requirements for recognition and cleavage of these two apparently unrelated substrates, an AP site and the *p*-BQ-dC (Figure 1), is clearly desirable. In the present work, we have attempted to approach this goal by using homogeneous HeLa AP endonuclease, recombinant HAP1, and its mutant proteins (Barzilay *et al*., 1995a,b; Rothwell & Hickson, 1996), and defined oligomers containing a single site-directed *p*-BQ-dC or an AP site (Chenna & Singer, 1995). The results indicate that the human AP endonuclease generally has the same requirements for the cleavage of an AP site and the benzene-derived exocyclic adduct, *p*-BQ-dC. Moreover, the enzyme appears to use the same active site to catalyze the cleavage of *p*-BQ-dC- and AP-containing DNA substrates.

MATERIALS AND METHODS

Materials. HeLa cells were obtained from Cell Culture Center, Endotronics Inc., Minneapolis (MN), a NIH-sponsored facility. Phosphocellulose P11 was obtained from Whatman. Blue Sepharose CL-6B, Mono-S HR5/5 FPLC, and Superdex 75 HR10/30 FPLC columns were all purchased from Pharmacia. FPLC was performed using a Pharmacia LKB system. The [γ-^{32}P]ATP (specific activity 6000 Ci/mmol) was purchased from Amersham. T4 polynucleotide kinase was purchased from USB. Uracil−DNA glycosylase (UDG) was obtained from Gibco, BRL. The gel filtration protein marker kit was from Sigma. Concentrators such as Centriplus-10 (MWCO: 10 000) and Centricon-10 (MWCO: 10 000) were purchased from Amicon. Dialysis membrane tubing (MWCO: 12−14 000) was from Spectrum Medical Industries. Sep-Pak columns were from Waters. OPC cartridges were from Applied Biosystems.

Oligonucleotide Substrates. The sequences of oligonucleotides used as substrates for examining various human repair activities are listed in Table 1. This includes substrates for 3-methyladenine (m^3A)−DNA glycosylase (sequences 4, 6, 8), 3,N^4-etheno-C (ϵC)−DNA glycosylase (sequence 5), uracil−DNA glycosylase (sequence 3), G/T mismatch−DNA glycosylase (sequence 9), and thymine glycol−DNA glycosylase (no. 11).

The synthesis of 3,N^4-*p*-BQ-dC, its phosphoramidite, and the site-directed 25-mer oligonucleotide (Table 1, sequence 2) were described by Chenna and Singer (1995). Each of the oligomers in Table 1 was annealed to a universal complementary 25-mer strand, 5′-ATTCGAGCTCGGTACCCGCTAGCGG-3′. Note that the base opposite each modified base is the correct wild-type one except that the base opposite T in sequence 9 was G, resulting in a G·T mismatch in the duplex. The 5-mer and 7-mer oligomer size markers used in the nicking assay were machine synthesized and purified by OPC cartridges.

For preparing the thymine glycol-containing DNA substrate, pBR322 plasmid DNA was treated with osmium tetraoxide (OsO_4) as described by Melamede *et al*. (1994).

The double-stranded 25-mer oligonucleotide containing a single AP site was prepared as follows: the dU-containing oligomer (Table 1, sequence 3) was treated with UDG (0.002−0.016 unit) for 20 min at 37 °C, resulting in an AP site at position 8 from the 5′ end. The UDG concentrations were chosen on the basis of obtaining greater than 99% removal of the uracil residual and subsequent conversion to an AP site (data not shown).

DNA Nicking Assay. The oligonucleotides were 5′-end labeled with [γ-^{32}P]ATP and annealed to a complementary oligonucleotide as described by Rydberg *et al*. (1991). The standard nicking assay used to examine the various enzymatic activities was carried out essentially as described by Rydberg *et al*. (1991, 1992). For testing purified column fractions and recombinant HAP1 proteins, BSA and $MgCl_2$ were routinely added to the reaction at various concentrations.

Calculation of the specific nicking activity of *p*-BQ-dC active fractions during protein purification and in other enzymatic assays was carried out as follows: The intact 25-mer and ^{32}P-labeled 7-mer fragment resulting from cleavage were scanned and integrated using a Hoefer Model GS300 densitometer connected to a Hoefer Model GS 350 data system. The percentage of cleavage for each damage-containing oligonucleotide was expressed as the percentage of nicking efficiency for the enzyme.

Enzymatic reactions to measure kinetic parameters of HAP1 protein for the *p*-BQ-dC- and AP site-containing substrates were performed as follows: Varying amounts of ^{32}P-labeled modified 25-mer oligonucleotide (6.6−46 nM) were incubated with HAP1 (216 pM for *p*-BQ-dC activity and 54 pM for AP activity) in 10 μL of 25 mM Hepes−KOH, pH 7.0, 0.5 mM dithiothreitol (DTT), 0.5 mM spermidine, 0.2 mM $MgCl_2$, 500 μg of BSA, and 10% glycerol for 5 min at 37 °C. After gel electrophoresis, the bands corresponding to substrate and product were excised and the radioactivity counted in a Beckman LS 1701 scintillation counter. The apparent K_m and $K_{cat.}$ values were determined from the Lineweaver−Burk plots.

For the study of metal ion requirements of the human AP endonuclease for *p*-BQ-dC and AP site-nicking activities, the modified templates were 5′-end-labeled with [γ-^{32}P]ATP in a modified kinase buffer (low Mg^{2+}) containing 50 mM Hepes−KOH, pH 7.5, 10 mM β-mercaptoethanol, and 0.2 mM $MgCl_2$. The annealing was performed in a buffer containing 10 mM Hepes−KOH, pH 7.5 and 100 mM NaCl. The final Mg^{2+} background level in the enzymatic reaction assay was 2.5 μM. The reaction buffer had a pH of 7.0, which was used to diminish nonenzymatic cleavage of the AP site.

For testing for any thymine glycol−DNA glycosylase activity, the OsO_4-treated pBR322 plasmid was incubated with column fractions in a buffer containing 35 mM Hepes−KOH, pH 7.8, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol. The mixture was resolved on a 1% agarose

Table 1: Synthetic 25-mer Oligonucleotides and Modifications

oligonucleotide	modification
1. 5′-CCGCTAGCGGGTACCGAGCTCGAAT-3′	none
2. 5′-CCGCTAG-***p*-BQ-C**-GGGTACCGAGCTCGAAT-3′	3,N^4-benzetheno-dC
3. 5′-CCGCTAG-**U**-GGGTACCGAGCTCGAAT-3′	deoxyuridine
4. 5′-CCGCT-ϵ**A**-GCGGGTACCGAGCTCGAAT-3′	1,N^6-etheno-dA
5. 5′-CCGCTAG-ϵ**C**-GGGTACCGAGCTCGAAT-3′	3,N^4-etheno-dC
6. 5′-CCGCTA-ϵ**G**-CGGGTACCGAGCTCGAAT-3′	1,N^2-etheno-dG
7. 5′-CCGCTA-**propanoG**-CGGGTACCGAGCTCGAAT-3′	1,N^2-propano-dG
8. 5′-CCGCT-**I**-GCGGGTACCGAGCTCGAAT-3′	deoxyinosine
9. 5′-CCGCTAG-**T**-GGGTACCGAGCTCGAAT-3′	G·T mismatch
10. 5′-CCGCTA-**8-oxo-G**-CGGGTACCGAGCTCGAAT-3′	7,8-dihydro-8-oxo-dG
11. pBR322 plasmid treated with OsO_4[a]	thymine glycol

[a] Osmium tetraoxide.

Table 2: Substitutions Made in the Mutant HAP1 Proteins

mutant protein	substitution	reference
E96A	glutamate → alanine	Barzilay *et al*., 1995a,b
D210N	aspartate → asparagine	unpublished
N212A	asparagine → alanine	Rothwell & Hickson, 1996
N212Q	asparagine → glutamine	Rothwell & Hickson, 1996
D219A	aspartate → alanine	Barzilay *et al*., 1995b
H116N	histidine → asparagine	unpublished

gel. DNA nicking could be determined by the conversion of supercoiled plasmid into either nicked circular or linear forms.

Purification of HeLa AP Endonuclease. This protein was purified from HeLa S3 whole cell extracts. All steps were performed at 0−4 °C, and at most of the steps the ^{32}P-labeled oligonucleotide substrates listed in Table 1 were used to monitor human AP endonuclease activity and various other enzymatic activities. The cell-free extract and 65% $(NH_4)_2SO_4$ precipitate were prepared essentially as previously described (Rydberg *et al*., 1991, 1992). The sample was then dialyzed to remove salt and passed through a phosphocellulose P11 column. The active fractions nicking *p*-BQ-dC from the column were pooled, desalted, and applied to a Blue Sepharose CL-6B column. In the next two steps, the active fractions were passed through a Mono-S HR5/5 FPLC column twice, using two different gradients and flow rates.

For further purification and determination of the native size of the desired protein, the above protein fraction was applied to a Superdex 75 HR 10/30 FPLC gel filtration column. The peak active fraction was shown to be homogeneous on silver stained SDS−PAGE gels (Hang *et al*., 1996b) and used in this study.

Recombinant HAP1 and Mutant Proteins. Site-directed mutagenesis of the HAP1 cDNA was carried out by the PCR-based method of Landt *et al*. (1990), as described by Barzilay *et al*. (1995a,b) and Rothwell and Hickson (1996) (Table 2). Purification of wild-type HAP1 and mutant HAP1 proteins, D219A, E96A, and H116N, was carried out as described in Barzilay *et al*. (1995a). Briefly, IPTG was used to induce HAP1 protein expression in BL21 (DE3) cells from the T7 promoter of the pT7-7 plasmid. HAP1 proteins were purified from a 70% $(NH_4)_2SO_4$ precipitate of soluble proteins by chromatography using phosphocellulose P11 (from which HAP1 eluted at approximately 550 mM NaCl) and FPLC phenylsuperose [from which HAP1 eluted at 50 mM $(NH_4)_2SO_4$].

The mutant HAP1 proteins D210N, N212A, and N212Q were purified from the same *E. coli* strain following IPTG-induced expression from the pET146 plasmid (Invitrogen). These hexahistidine-tagged proteins were purified by chromatography on phosphocellulose P11, as described above, and a nickel chelate affinity resin, using 0.5 M imidazole to elute bound proteins. All recombinant HAP1 proteins were ≥95% pure, as judged by SDS−PAGE analysis.

Molecular Modeling. Energy minimization of the 25-mer double helices was carried out by AMBER force field in HyperChem (Hypercube). An AP site and the *p*-BQ-C were manually formed from the cytosine base in the position 8 of the strand (Table 1) of a B-geometric duplex before optimization.

RESULTS

Substrate Specificity of the Major Human AP Endonuclease. The substrate range of the major human AP endonuclease was studied using defined site-specific oligonucleotides containing structurally related exocyclic derivatives and some other base modifications. The purified enzyme does not act on other exocyclic adducts such as 1,N^6-ϵdA, 3,N^4-ϵdC, 1,N^2-ϵdG, and 1,N^2-propano-dG. In addition, other base damages such as thymine glycol and 8-oxo-dG are also not substrates for cleavage by the enzyme (data not shown).

In this study of *p*-BQ-dC cleavage, the enzyme repairs a double-stranded oligonucleotide substrate. The enzyme does not act on an unmodified identical duplex oligonucleotide, and no detectable exonuclease activity is observed under the conditions used, in agreement with the majority of the published data (Kane & Linn, 1981; Robson *et al*., 1991; Barzilay *et al*., 1995b). By using complementary strands with any of the four bases opposite the *p*-BQ-dC adduct, nicking of oligonucleotides containing all four *p*-BQ-dC base pairs was detected, but the extent of nicking efficiency varied greatly (Figure 2). The maximal nicking activity was found with a *p*-BQ-dC·G pair, while less than 20% of the maximal activity was found with a *p*-BQ-dC·C pair.

Enzymatic and Biochemical Properties of the AP Endonuclease toward the p-BQ-dC Adduct and an AP Site. A DNA nicking assay based on a ^{32}P-labeled 25-mer defined sequence containing either a single *p*-BQ-dC or dU at the same position (Table 1) was utilized for quantitative measurement of the *p*-BQ-dC and AP activities. In either cases, the base opposite *p*-BQ-dC or dU was a G. The dU-containing oligomer was treated with uracil−DNA glycosylase prior to HAP1 cleavage under reaction conditions that could produce >99% of the AP site.

The comparative kinetic analysis of *p*-BQ-dC and AP site cleavage by HAP1 protein was carried out by varying ^{32}P

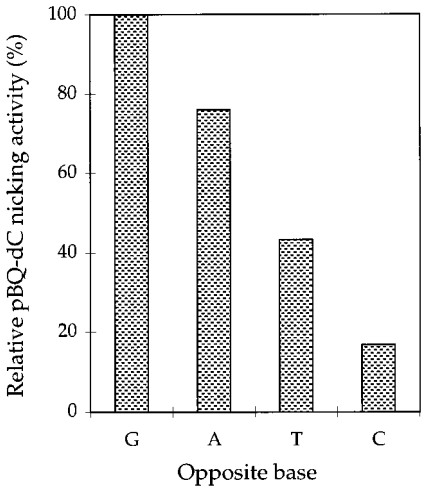
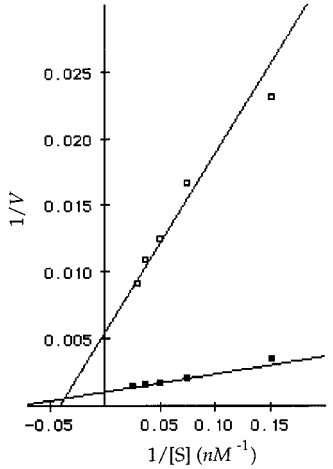


FIGURE 2: Effect of the base placed opposite *p*-BQ-dC on the *p*-BQ-dC endonuclease activity of HeLa AP endonuclease. Oligonucleotide 2 in Table 1 was annealed to complementary strands with each of the four bases opposite the *p*-BQ-dC adduct and incubated with purified *p*-BQ-dC endonuclease (2 ng) at 37 °C for 1 h. The *p*-BQ-dC activity of *p*-BQ-dC•G was maximal and arbitrarily assigned as 100%, and the activities of the other three pairs were then calculated as percent of this maximal activity. These results do not imply that any base forms conventional Watson−Crick base-pairs with *p*-BQ-dC.



Comparative Kinetic Analysis of Cleavage of *p*-BQ-dC and AP Site by HAP1 Enzyme

Substrate	K_m (nM)	K_{cat} (min^{-1})	K_{cat}/K_m ($min^{-1}nM^{-1}$)
p-BQ-dC	24.9	6.6	0.3
AP site	13.2	34.7	2.5

FIGURE 3: Representative Lineweaver−Burk plots of the initial velocities of the *p*-BQ-dC and AP endonuclease activities of HAP1 enzyme. The reactions were performed in parallel in a standard reaction mixture (see Materials and Methods) with varying oligomer substrate concentrations (6.6−46 nM) for 5 min at 37 °C. The kinetic parameters listed in the table were calculated from the plots shown above. Reaction velocity (V) was calculated as picomoles of the ^{32}P-labeled 7-mer cleavage product formed per minute per nanogram of enzyme. *p*-BQ-dC, □; AP site, ■.

end-labeled substrate concentrations. The reaction velocity was calculated as picomoles of cleavage product produced per minute per nanogram of protein. As shown in Figure 3, the *p*-BQ-dC was cleaved less efficiently than an AP site, as evidenced by the values of specificity constants (K_{cat}/K_m) for *p*-BQ-dC and AP site of 0.3 and 2.5 (min^{-1} nM^{-1}), respectively. The difference was mainly due to a 5-fold decrease in the $K_{cat.}$ of HAP1 on the *p*-BQ-dC substrate. The K_m for AP site cleavage was approximately half of that for *p*-BQ-dC cleavage (Figure 3).

The AP endonuclease activity of the HAP1, like its *E. coli* homologue, exonuclease III, is known to require divalent metal ions. In this study, a parallel comparison was made of the relative activities of the isolated HeLa AP endonuclease toward *p*-BQ-dC and an AP site in the presence of each individual metal ion (Figure 4). Similar to its AP activity, the *p*-BQ-dC activity of the enzyme also requires Mg^{2+}, Mn^{2+}, or Zn^{2+}, which can substitute for each other. EDTA inactivates both activities (Figure 4A). The two activities were active over a broad range of Mg^{2+} concentration but both showed maximal activities at 0.05−0.1 mM (Figure 4B). Note that the enzyme concentration for assaying for AP activity was 15-fold lower than that for *p*-BQ-dC activity in Figure 4B−F. In the case of Mn^{2+}, a similar pattern of dependence was observed with peak activities clustered at 0.05−0.1 mM, except that higher AP activity (Figure 4C) was obtained. Figure 4D shows that Zn^{2+} had almost the same stimulatory effect on *p*-BQ-dC and AP activities. The enzyme apparently dose not require Ca^{2+} (Figure 4E) or KCl (Figure 4F). Since the activity toward the two substrates was different, absolute comparisons cannot be made of the peak heights.

The enzyme is most active between pH 5.5 and 6.5 for both substrates (Figure 5) and not at what is considered physiological conditions. As with other comparisons of the two substrates, at the same protein concentration, the AP activity was higher than *p*-BQ-dC activity.

p-BQ-dC and AP Endonuclease Activities of Mutant HAP1 Proteins. Six mutant HAP1 proteins containing single amino acid substitutions were analyzed by measuring their rates of nicking activities toward the *p*-BQ-dC and AP site, as compared to that of the corresponding activities of the wild-type HAP1 protein. The substitutions were made at sites of conserved amino acids by site-directed mutagenesis (Table 2).

As shown in Figure 6, there is either concomitant reduction in both *p*-BQ-dC and AP endonuclease activity or complete loss of both measurable activities. Note that 4-fold higher concentrations of mutant proteins were used than the wild-type HAP1 protein for assaying both activities. The D219A, E96A, and N212Q mutant proteins showed reduced, but not totally abolished, cleavage activities toward both the *p*-BQ-dC adduct (4.7-, 26.9-, and 32.6-fold reduction, respectively) and the AP site (25.9-, 33.1-, and 37.2-fold less), as compared to that of the wild-type HAP1 protein, while N212A and D210N completely lost both their enzymatic activities toward these two substrates, under the conditions used (Figure 6). The mutant H116N retained similar levels of activities toward the *p*-BQ-dC and AP sites compared to wild-type HAP1 activity (data not shown).

It has been proposed that HAP1 contains a single metal ion bound to Glu 96 that is part of the active site (Barzilay *et al.*, 1995a). The E96A mutant has been previously shown to have an altered Mg^{2+} ion dependence of AP endonuclease activity (Barzilay *et al.*, 1995a). In order to further examine whether this substitution would also affect the metal ion requirement for the *p*-BQ-dC activity, we compared the effects of varying the Mg^{2+} concentration on the *p*-BQ-dC activity of E96A mutant and wild-type HAP1 protein.

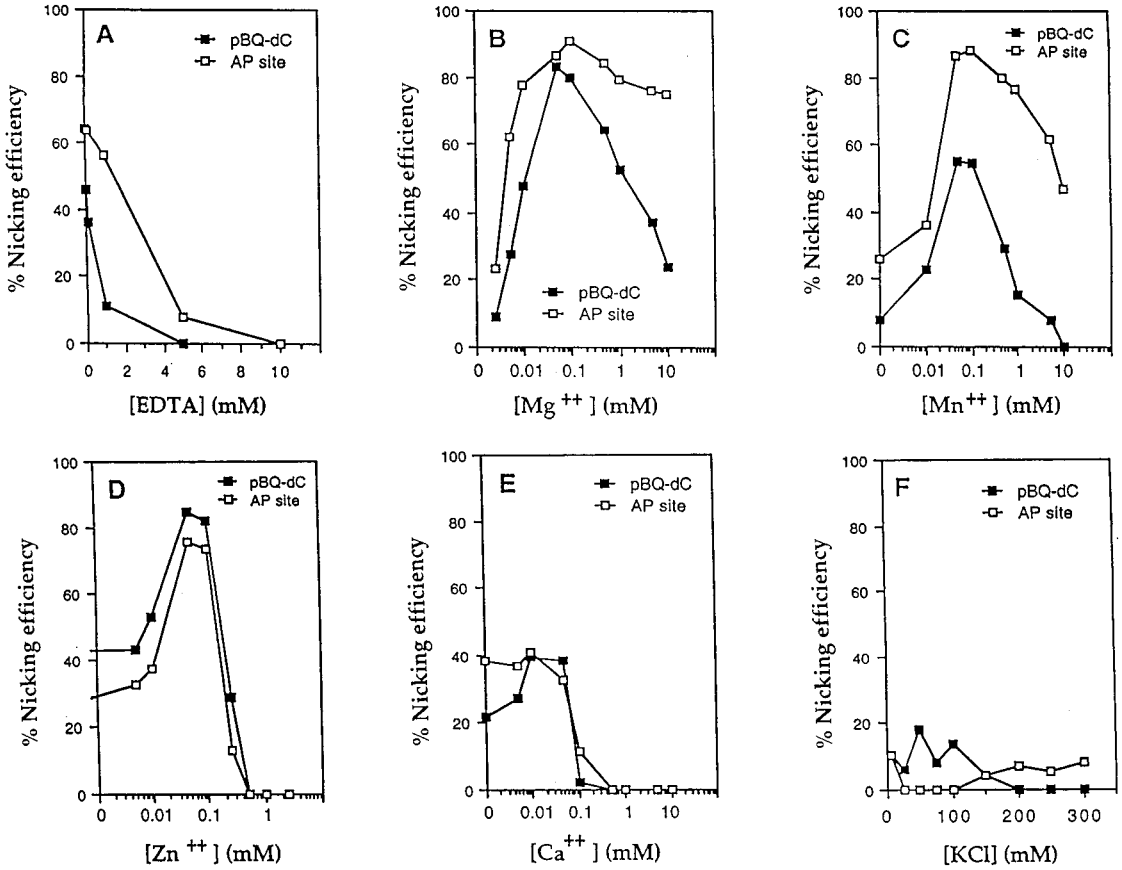


FIGURE 4: Comparison of metal ion requirements for *p*-BQ-dC activity (■) and AP activity (□) of the purified enzyme using as substrates the 25-mer *p*-BQ-dC-containing oligomer and the UDG-treated dU-containing oligomer. All the reactions illustrated in this figure were carried out at 37 °C for 30 min in the assay buffer of pH 7.0 with oligonucleotide substrates labeled under low Mg^{2+}-kinase buffer, as described under Materials and Methods. Therefore, all the reactions contained 2.5 μM $MgCl_2$ as background. (A) EDTA chelation: 2 ng of the *p*-BQ-dC endonuclease was incubated with *p*-BQ-dC or AP oligomer in the presence of 0.5 mM $MgCl_2$ with increasing EDTA concentration. In all other panels from B to F, 2 ng of the AP endonuclease was used for assaying for *p*-BQ-dC nicking and 0.13 ng for AP activity. The metal ions used are indicated below each panel.

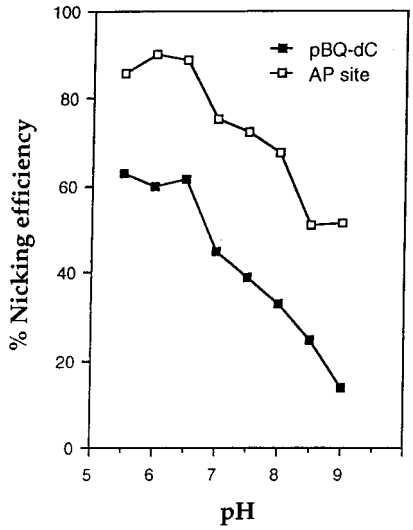


FIGURE 5: Effect of pH on the *p*-BQ-dC and AP endonuclease activities of the HeLa AP endonuclease. The *p*-BQ-dC oligonucleotide and UDG-treated dU oligonucleotide were reacted with AP endonuclease (1.3 ng) in a potassium phosphate buffer with varying pHs from 5.5 to 9.5, as indicated. The reaction was carried out at 37 °C for 30 min in the presence of 0.5 mM $MgCl_2$.

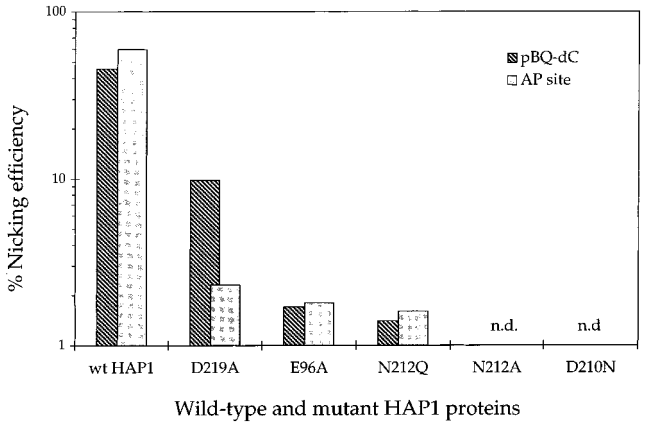


FIGURE 6: Comparison of the endonuclease activity toward *p*-BQ-dC and the AP site using wild-type and mutant HAP1 proteins. In these experiments, 50 and 0.5 ng of wild-type HAP1 was used for testing the *p*-BQ-dC activity and the AP activity, respectively. Similarly, 200 and 2 ng of various mutant proteins (as shown below each bar) was used for assaying their activities against *p*-BQ-dC and AP site, respectively. The reaction conditions were the same as described in Figure 4, and the data were taken from the rate of cleavage at a 5 min point in a time course plot. The specific amino acid exchanges are given for each mutant in Table 2. Note that the ordinate is a log scale. n.d.: not detected.

Indeed, the E96A mutant also showed a similar alteration of the Mg^{2+} requirement to that for the AP endonuclease activity. As shown in Figure 7, the E96A mutant requires higher Mg^{2+} for *p*-BQ-dC activity.

Molecular Modeling of Oligonucleotides Containing the p-BQ-dC or an AP Site. The mechanisms of substrate recognition for the multifunctional AP endonucleases have been the subject of investigation for a long time but are still

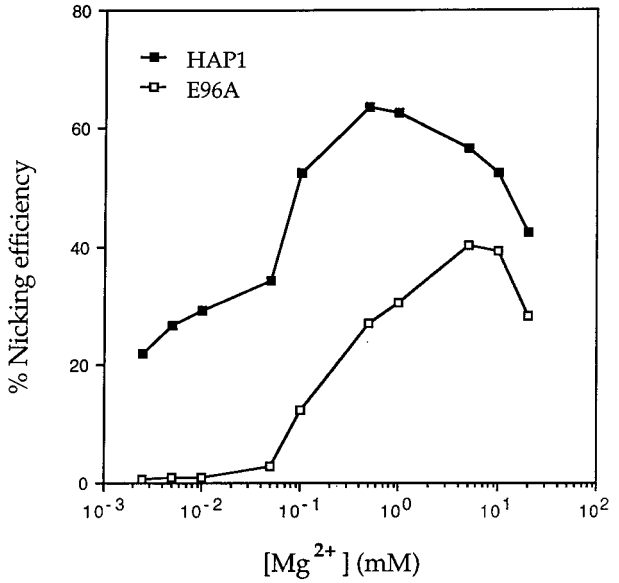


FIGURE 7: Effect of Mg^{2+} concentrations on the *p*-BQ-dC endonuclease activity of wild-type HAP1 and E96A proteins. Forty nanograms of wild-type HAP1 protein and 600 ng of E96A protein were incubated with *p*-BQ-dC-containing 25-mer oligonucleotide at 37 °C for 30 min. The background level of Mg^{2+} in the reaction mixture was 2.5 μM.

not solved at the molecular level. The finding that *p*-BQ-dC is a new substrate for these enzymes further complicates this situation. Since at this moment no structural information is available for the *p*-BQ-dC adduct, we carried out simple molecular modeling using the Hyperchem program, for a preliminary comparison of the effect of *p*-BQ-dC and an AP site on local DNA structure. Figure 8 shows the energy-minimized conformations of 5 base-pairs, from nucleotides 6−10 of the annealed 25-mer oligomers (Table 1). These sequences contain either an unmodified cytosine (A), or a *p*-BQ-C base (B), or a regular AP site (C), all at position 8. As shown in Figure 8, the duplex at the abasic site "collapses", whereas the bulky *p*-BQ-C creates a vertical "bubble". The finding described here for the structural change caused by the AP site is similar to previously reported data on the conformation of DNA at an apyrimidinic site obtained through NMR studies (Kalnik *et al*., 1988; Cuniasse et al., 1990; Withka et al., 1991; Goljer et al., 1995). The presence of an AP site changes the conformation of at least one base-pair on each side, whereas the presence of *p*-BQ-dC changes the conformation of at least two base-pairs in each direction. Therefore, *p*-BQ-dC causes a different conformational change than an AP site in the duplex. The detailed conformation changes caused by a single *p*-BQ-dC are currently under investigation.

DISCUSSION

Exocyclic DNA adducts represent a large group of potentially mutagenic and carcinogenic DNA lesions derived from variety of exogenous as well as endogenous chemicals (Singer & Bartsch, 1986; Marnett & Burchan, 1993). A DNA glycosylase-mediated base excision mechanism has been reported for the repair of some of these adducts such as etheno bases (Singer, 1996; Habraken *et al*., 1991; Matijasevic *et al*., 1992; Singer *et al*., 1992; Dosanjh *et al*., 1994; Hang *et al*., 1996a). For this reason, we first examined during protein purification whether any of those previously known DNA glycosylases was responsible for the *p*-BQ-dC nicking activity found in HeLa cell extracts (Chenna *et al*., 1995). We have found no overlap in substrate specificity between the *p*-BQ-dC nicking activity and various DNA glycosylase activities tested. Instead, the *p*-BQ-dC-containing oligomer is cleaved by 5′ AP endonucleases (Class II) (Hang *et al*., 1996b). This apparently adds a new mechanism for the repair of the exocyclic DNA lesions, extends the area of investigation of AP endonucleases to a new class of substrates, and has implications for their mode of action.

This finding raises an interesting question about the requirements for the recognition and cleavage of the adduct, which is apparently structurally unrelated to the principal substrate of these enzymes, an AP site. The results of this present work provide a parallel comparison of the properties and requirements of these two activities in an attempt to gain insight into the above important questions.

"Class II" AP endonucleases are generally relatively small (25−40 kDa) and monomeric proteins (Demple & Harrison, 1994; Barzilay & Hickson, 1995). A characteristic feature of these proteins is their multifunctional activities, ranging from those toward regular or synthetic AP sites to 3′ blocking lesions (3′-phosphate, 3′-deoxyribose 5′-phosphate, or 3′-phosphoglycoaldehyde). Besides these properties, each enzyme also shows other distinct activities. For example, the *E. coli* major AP endonuclease, exonuclease III, has associated 3′ → 5′ exonuclease activity, while HAP1 evolved a novel activity that mediates the reductive activation of oxidized transcription factors ("redox" function). This latter activity is structurally and functionally distinct from the repair activities of HAP1 enzyme, with cysteine 65 identified as the active site for the redox activity (Xanthoudakis & Curran, 1992; Xanthoudakis *et al*., 1992; Walker *et al*., 1993).

In this current work, we focused on whether the HAP1 needs the same requirements for the AP function and the newly identified substrate, *p*-BQ-dC. We used a DNA nicking assay based on two defined 25-mer oligonucleotides containing either *p*-BQ-dC or an AP site (Table 1). The two lesions studied are in the same position in the oligonucleotides and thus have identical 3′ and 5′ sequences. Any differences found in repair may reflect conformational differences conferred by each adduct (Figure 8). The major difference found in all comparisons made in this study was the approximately 8-fold higher specific activity toward an AP site compared to *p*-BQ-dC. This is mainly a result of the decrease in the apparent $K_{cat.}$ for *p*-BQ-dC (Figure 3). In a similar case, Kow (1989) reported that in a series of AP substrates there were large quantitative differences in the activity of exonuclease III. He observed that the apparent K_m for different *O*-alkylhydroxylamine residues was not affected by the particular *O*-alkylhydroxylamine residue substituted; however, the apparent V_{max} decreased as the size of the residue increased.

Weiss (1976) proposed that exonuclease III possesses a single active site for the catalysis of various diverse substrates then used. This theory has been confirmed by 3-D crystallographic studies on this enzyme (Mol *et al*., 1995). Recently, Barzilay et al (1995a,b) have provided evidence for the presence of a single catalytic active site in the human AP endonuclease, HAP1, for all known repair activities. Moreover, structural studies on HAP1 (Gorman *et al*., manuscript in preparation) have substantiated this. In this

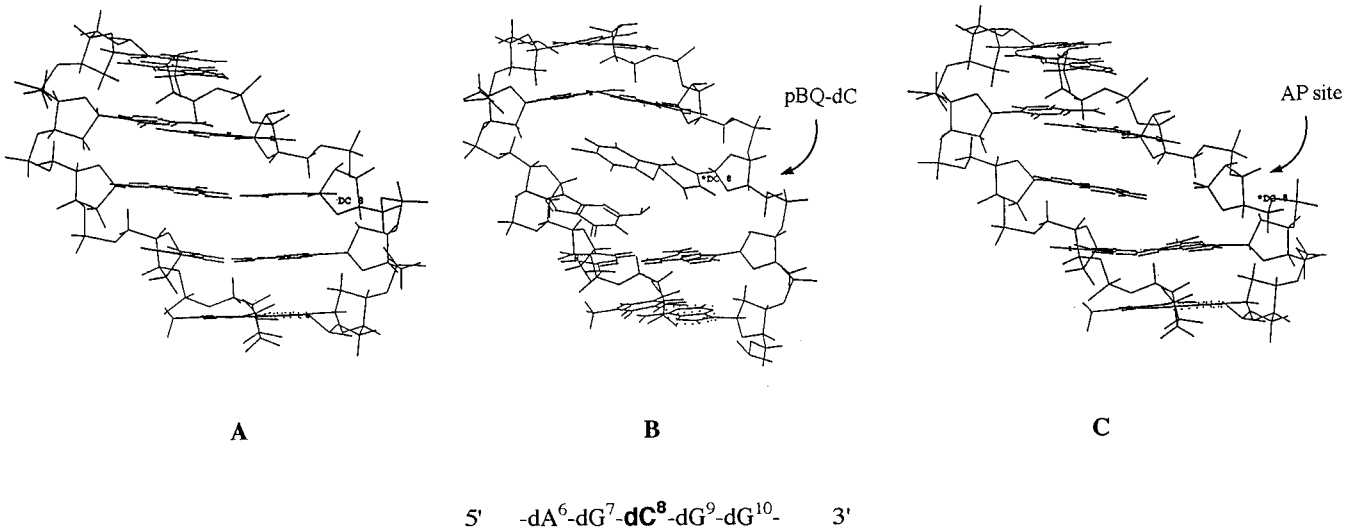


FIGURE 8: Energy-minimized conformations of five base-pairs (positions 6−10) of 25-mer duplexes containing at position 8: (a) unmodified dC (A), (b) *p*-BQ-dC (B), and (c) an AP site (C). The arrows point to the central base. The oligonucleotide sequences used are given as 1−3 in Table 1.

work, we have addressed whether HAP1 utilizes the same catalytic active site for the *p*-BQ-dC endonuclease activity by using six HAP1 mutants containing single amino acid substitutions. Asn 212 residue is essential for the AP recognition/binding by HAP1 (Rothwell & Hickson, 1996), which is in agreement with structural data indicating both that this residue is in the active site of HAP1 and that the equivalent residue in exonuclease III, Asn 153, is an active-site residue (Mol *et al*., 1995). Asp 219 is also known to be critical for the HAP1 binding and enzymatic activity, as shown by the marked reduction in both AP and RNase H activity after replacing Asp 219 with alanine (D219A) (Barzilay *et al*., 1995b). Glu 96 in HAP1 corresponds to Glu 34 in exonuclease III that have been shown to play a metal ion binding role in these enzymes and to facilitate enzymatic catalysis (Mol *et al*., 1995). Glu 96 has been previously confirmed to play a vital role in AP activity and metal ion binding in HAP1 (Barzilay *et al*., 1995a).

Our results showed that D219A, E96A, and N212Q had a similar reduction in *p*-BQ-dC endonuclease activity as compared with their AP activity (Figure 6), while D210N and N212A completely lacked any detectable enzymatic activities toward the two substrates. Another non-active-site substitution mutant, H116N, did not affect *p*-BQ-dC or AP activity. This parallelism strongly supports the hypothesis that *p*-BQ-dC endonuclease activity of the HAP1 is also mediated via the same catalytic active site as the AP function.

HAP1 is a divalent cation-dependent AP endonuclease (Barzilay *et al*., 1995a). Apparently, its *p*-BQ-dC activity has similar requirements for divalent metal ions (Figure 4) in terms of range and optimal spectra. To examine this further, we studied the effect of Mg^{2+} on the *p*-BQ-dC activity of E96A, a crucial residue involved in metal ion binding in HAP1 (Barzilay *et al*., 1995a; Gorman *et al*., manuscript in preparation). In addition to its markedly reduced activities toward *p*-BQ-dC and the AP site (Figure 6), the *p*-BQ-dC activity of the E96A protein also showed an altered Mg^{2+} ion dependence (Figure 7), which is very similar in pattern to that found for the AP activity (Barzilay *et al*., 1995a). From these data, we conclude that the *p*-BQ-dC cleavage requires the same active site as that for the AP cleavage. This conclusion can only be confirmed when the cocrystal structure of HAP1 with the *p*-BQ-dC adduct-containing DNA is available.

The presence of a single catalytic active site suggests a common structural feature for the *p*-BQ-dC adduct and an AP site. As a matter of fact, so far there is no clear generality in recognition signals for the HAP1 or its bacterial homologue, exonuclease III (Singer & Hang, 1997). In the case of *p*-BQ-dC, our preliminary molecular modeling has showed that the general conformational change differs with *p*-BQ-dC, causing more local perturbation (Figure 8). Clearly, there cannot be Watson−Crick base-pairing in either case. Considering the data that HAP1 requires a double-stranded substrate containing *p*-BQ-dC and also differentially cleaves the *p*-BQ-dC paired with different opposite bases (Figure 2), we believe that the recognition signal for repair is not the primary structure of the adduct, but rather it is the localized effect of *p*-BQ-dC on the nucleic acid structure that plays a crucial role in enzyme recognition.

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