# Human Endonuclease III Acts Preferentially on DNA Damage Opposite Guanine Residues in DNA†

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ABSTRACT: The human endonuclease III homologue (hNTH1) removes premutagenic cytosine damage from DNA. This includes 5-hydroxycytosine, which has increased potential for pairing with adenine, resulting in C → T transition mutations. Here we report that hNTH1 acts on both 5-hydroxycytosine and abasic sites preferentially when these are situated opposite guanines in DNA. Discrimination against other opposite bases is strongly dependent on the presence of magnesium. To further elucidate this effect, we have introduced mutations in the helix−hairpin−helix domain of hNTH1 (K212S, P211R, +G212, and ΔP211), and measured the kinetics of 5-hydroxycytosine removal of the mutants relative to wild type. The K212S and ΔP211 (truncated hairpin) mutant proteins were both inactive, whereas the extended hairpin in the +G212 mutant diminished recognition and binding to 5-hydroxycytosine-containing DNA. The P211R mutant resembled native hNTH1, except for decreased specificity of binding. Despite the altered kinetic parameters, the active mutants retained the ability to discriminate against the pairing base, indicating that enzyme interactions with the opposite strand relies on other domains than the active site helix−hairpin−helix motif.

DNA suffers from continuous exposure to reactive oxygen species in aerobic organisms. This exposure may lead to oxidative DNA damage, which is assumed to be involved in aging and carcinogenesis (*1*, *2*). Potent oxidizing agents readily attack DNA bases and more than 100 different products arising from these have been described (for review, see ref *3*). Many of these lesions may miscode or block replication. Among the miscoding lesions formed, the potentially mutagenic cytosine derived lesions, 5-hydroxycytosine (5ohC),[1] 5-hydroxyuracil, and uracil glycol, have been paid special interest. The high abundance of these damages in mammalian cells makes them interesting due to the relative high frequency of C−T transition mutations that occurs from oxidative DNA damaging agents (*4*−*6*).

Oxidative DNA base damage is normally repaired by the base excision repair (BER) pathway (*7*). DNA glycosylases initiate the pathway by removal of the damaged base. Most oxidized bases are removed by DNA glycosylases with a combined apurinic/apyrimidinic (AP) lyase activity. In *Escherichia coli*, endonuclease III (Nth) recognizes a variety of pyrimidine base damages. Endonuclease III has been conserved from *E. coli* to human cells. The proteins contain the helix−hairpin−helix (HhH) domain, thought to be required for DNA binding, as well as a [4Fe-4S] cluster. The human endonuclease III homologue (hNTH1) has been cloned and characterized (*8*−*11*) and has a substrate specificity that closely resembles bacterial Nth. hNTH1 (33.5 kDa) is significantly larger than Nth (24 kDa) and contains an N-terminal domain that is involved in intracellular transport (*11*).

The three-dimensional structure of Nth has been determined (*12*, *13*). The protein consists of two independent domains that are connected by the HhH domain. The C-terminal end carries the [4Fe-4S] cluster implicated in proper binding to DNA. The two domains form a pocket into which the base is suggested to flip during the reaction. The residues $D^{138}$ and $K^{120}$ play central roles in the reaction, which involves formation of a covalent Schiff base intermediate. This intermediate is released by a coupled $\beta$-elimination reaction resulting in strand cleavage. Both $D^{138}$ and $K^{120}$ are conserved throughout the endonuclease III class of enzymes (*8*), suggesting a common reaction mechanism. The HhH domain has been implicated in DNA recognition and binding, and small amino acid variations within the region have dramatic effects on the selectivity and function of the glycosylase. For example, DNA glycosylases without an associated AP lyase activity such as AlkA or MutY (*E. coli*)

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[1] Abbreviations: 5ohC, 5-hydroxycytosine; AP, abasic site; BER, base excision repair; Nth, endonuclease III; hNTH1, human endonuclease III homologue 1; HhH, helix-hairpin-helix; faPy, formamidopyrimidine.

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harbor a W and S, respectively, in place of the conserved K (*14*).

The efficiency of hNTH1 is rather low as compared to the bacterial enzyme (*9*), but the activity is stimulated by the nucleotide excision repair protein XPG in vitro (*15*, *16*). Another repair protein that stimulates DNA glycosylase activity is the human endonuclease Ape1/HAP1/APEX/Ref1 (*17*−*20*), which has been shown to promote the TDG DNA glycosylase turnover. Additionally, the turnover of TDG glycosylase depends on magnesium and the identity of the pairing base (*21*). To further characterize hNTH1 with respect to DNA damage recognition and removal, we have examined the putative role of magnesium in the repair of two different lesions, 5ohC and an abasic site in different pairing contexts. The effect of adding Ape1 to the reactions was also investigated. Furthermore, HhH mutants were generated and characterized biochemically to evaluate the involvement of the HhH domain.

## MATERIALS AND METHODS

*Expression Clones, Mutant Generation, and Purification of Proteins.* Full-length hNTH1 cDNA cloned into the pGEX-2T vector (pGEX-hNTH1-11) as previously described (*11*), represents the wild-type hNTH1 protein. Four different mutant proteins with altered HhH motifs were created (Figure 3). The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce the desired changes, with the pGEX-hNTH1-11 construct as the parental vector according to the manufacturer's protocol. The oligonucleotides used to generate mutations were (from 5′ to 3′ end, hNTH1 sequence is shown for comparison):

GCGCTGCCGGGTGTTGGGCCCAAGATGGCACACCTGGCTATG (hNTH1)

GCGCTGCCGGGTGTTGGGCGCAAGATGGCACACCTGGCTATG (P211R)

GCGCTGCCGGGTGTTGGGCCCAGCATGGCACACCTGGCTATG (K212S)

GCGCTGCCGGGTGTTGGGCCCGGGAAGATGGCACACCTGGC (+G212)

GCGCTGCCGGGTGTTGGG___AAGATGGCACACCTGGCTATG (ΔP211)

The desired mutations were verified by sequence analysis. The fusion plasmids of wild-type hNTH1 and mutant proteins were transformed into *E. coli* strain AB1157 for expression. Expression and purification were performed essentially as described (*9*) with some modifications. The proteolysis was performed at room temperature using 8 U of thrombin (SIGMA) per milligram of fusion protein. After 1 h, 1 mM PMSF was added to the proteolysis mixtures, and the solutions were passed through a glutathione-Sepharose column. The eluates were dialyzed against phosphate buffer (100 mM $KHPO_4$, pH 6.6, 2 mM EDTA, 10% glycerol, 150 mM NaCl, 5 mM $\beta$-mercaptoethanol). The dialyzed proteins were applied on a MonoS column equilibrated with the same buffer, and eluted with a linear gradient from 150 to 750 mM NaCl. The full-length mutant proteins eluted from the column at around 650 mM NaCl. Truncated proteolysis products and uncleaved fusion proteins eluted earlier. The most concentrated fractions were added glycerol (30%) and stored at −20 °C. Ape1 protein was purified as described by Masuda et al. (*22*). Nfo protein was kindly provided by Magnar Bjørås.

*DNA Substrates.* $^3$H-formamidopyrimidine (faPy)-containing DNA was prepared as described previously (*23*). 5-hydroxycytosine 40-mer oligonucleotide with sequence 5′-AATTGCGATCTAGCTCGCCAG(5ohC)AGCGACCTTATCTGATGA-3′ was prepared as described (*5*), and its mass was verified by MALDI analysis. The oligonucleotide was $^{32}$P-end-labeled using [$\gamma$-$^{32}$P] ATP and T4 polynucleotide kinase (MBI Fermentas), and hybridized to complementary strands with either adenine or guanine pairing with 5ohC (5ohC-A and 5ohC-G duplexes, respectively).

Abasic DNA was generated similarly by $^{32}$P-end-labeling the 25-mer oligonucleotides (5′-GCTCATGCGCAGUCAGCCGTACTCG-3′; filled symbols), and (5′-GGCGGCATGACCCUGAGGCCCATC-3′; open symbols), and subsequently hybridizing these to complementary strands with adenine, cytosine, guanine, or thymidine opposite to uracil as described (*24*). AP DNA was generated by incubation with uracil DNA glycosylase (MBI Fermentas) for 30 min at 37 °C prior to use.

*Enzymatic Reactions.* Unless otherwise stated, all reactions were performed in reaction buffer: (70 mM MOPS, pH 7.5, 5% glycerol, 50 mM KCl, 0.01% BSA, and 1 mM dithiothreitol). Enzymes were diluted immediately before use in reaction buffer. faPy DNA glycosylase assays (50 $\mu$L) contained 0.5 $\mu$g of faPy-containing DNA (equivalent to 20 fmol of $^3$H-faPy lesions or 1000 DPM) and were incubated at 37 °C for 30 min. Released faPy residues were determined from the amount of ethanol-soluble radioactivity in the supernatant (*23*).

For the kinetic experiments, different amounts of 5-hydroxycytosine DNA were incubated with hNTH1 in reaction buffer (10 $\mu$L) at 37 °C for 20 min. Incision of the duplex with time was linear during this period. The reactions were stopped by the addition of 10 $\mu$L of formamide, and the incised products were separated from the 40-mer by 10% UREA−PAGE. The data were quantified by PhosphoImaging (Molecular Dynamics). Initial velocity was plotted against substrate concentration, and the $K_m$ value was subsequently determined from the plot corresponding to 0.5·*V*max on the abcissa.

The electrophoretic mobility shift assay (10 $\mu$L) contained 1 fmol of $^{32}$P-end-labeled 5ohC-oligonucleotide and 200 fmol of hNth protein in reaction buffer with 2 mM $MgCl_2$. The reactions were incubated on ice for 15 min prior to loading and separation on 10% nondenaturing PAGE. The gels were run at 80 V for 1 h at 4 °C, dried, and subjected to PhosphoImaging.

## RESULTS

*Incision of 5-Hydroxycytosine Is Dependent on Magnesium and the Opposite Base.* 5ohC is premutagenic and may miscode by pairing with adenine. Bases in a mispairing context with a potency of forming mutations are sometimes removed with low efficiency, such as 8-oxoG-A for the 8-oxoguanine DNA glycosylase homologues (*24*, *25*). A recent report has indicated that the activities of the endonuclease III enzymes also are dependent on the base in the opposite strand (*26*). The possible role of magnesium in discriminating 5ohC in different pairing contexts by hNTH1 was investigated. As shown in Figure 1, repair of 5ohC by hNTH1 was dependent on magnesium. Repair of a 5ohC opposite guanine was approximately 2-fold stimulated with increasing magnesium concentrations, whereas 5ohC paired

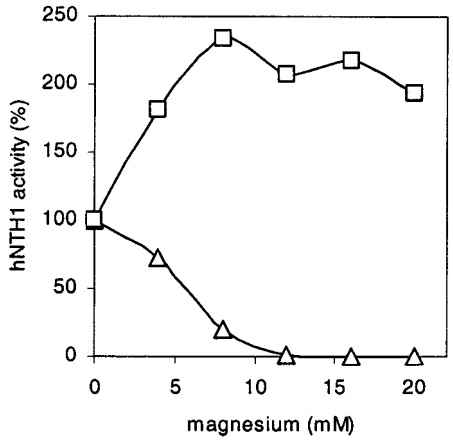


FIGURE 1: Effect of magnesium on the incision of 5ohC-DNA by hNTH1. 0.3 nM hNTH1 was incubated with 1.5 nM $^{32}$P-end-labeled 5ohC-A (△) or 5ohC-G (□) DNA duplexes for 20 min at 37 °C in reaction buffer containing different concentrations of $MgCl_2$. The products were separated by denaturing PAGE and analyzed by phosphoImaging. hNTH1 activity is calculated relative to the amount of incised substrate at 0 mM $MgCl_2$ (corresponds to 10% conversion of substrate duplex).

with adenine was poorly repaired with increasing magnesium concentrations. No incision of 5ohC opposite adenine was observed above 15 mM $MgCl_2$, when incision of 5ohC paired with guanine was optimal (Figure 1).

*hNTH1 Incises Lesions Positioned Opposite Guanine Most Efficiently.* In view of the discrimination in 5ohC repair, the optimal pairing for hNTH1 substrates was evaluated. 5ohC is formed in G−C base pairs, and pairing with adenine might induce conformational changes in the DNA that inhibit hNTH1 activity. To circumvent such problems, abasic DNA was chosen as substrate. Abasic (AP) sites are noncoding lesions and do not favor pairing to any specific base in human cells (*27*). DNA substrates where the AP site was positioned opposite guanine, adenine, thymine, or cytosine were prepared. The resulting duplexes, AP−G, AP−A, AP−T, and AP−C were incubated with hNTH1 in the presence of 10 mM $MgCl_2$. As shown in Figure 2A, incision of an AP site opposite guanine was at least 100-fold more efficient than for the other substrates. No difference in cleavage of different substrates could be observed in the absence of magnesium (data not shown), and the incision efficiency was calculated relative to 0 mM $MgCl_2$.

To test for possible effects of sequence context, AP-DNA duplexes with a different sequence were prepared with guanine or adenine opposite the AP site. As shown in Figure 2B, incision by hNTH1 of AP-sites opposite adenine is strongly suppressed also in this case. The different incision of the two AP−G duplexes with increasing magnesium concentrations may reflect that DNA sequence context has some effect on the hNTH1 activity, as is observed for other DNA glycosylases (*28*−*30*).

*Kinetics of 5-Hydroxycytosine Incision.* To further investigate the effect of magnesium on the hNTH1 activity, the kinetics of 5-hydroxycytosine incision was determined. EDTA is regularly used in characterization of DNA glycosylases. For comparison, the kinetics for 5ohC repair by hNTH1 was investigated in buffer containing either EDTA or magnesium (2 mM). Only a minor effect of the opposite base was observed in the absence of magnesium (1.8-fold), whereas 5ohC opposite guanine was incised approximately 15 times more efficiently than opposite adenine in 2 mM magnesium (Table 1). The preferential repair of 5ohC-G in the presence of magnesium resulted from a decreased $K_m$ value and to a lesser extent increased $k_{cat}$ value as compared to 5ohC-A, implying that 5ohC-G is better recognized by hNTH1 than 5ohC-A.

*Expression, Purification, and faPy DNA Glycosylase Activity of the hNTH1 Mutant Proteins.* The HhH domain has been implicated in DNA binding and damage recognition. To test whether this domain is critical for the discriminative recognition of 5ohC-A versus 5ohC-G by hNTH1, mutations were introduced into the hairpin region, and the resulting mutant proteins were characterized (Figure 3). Two of the mutations involved either increasing (+G212) or decreasing (ΔP211) the length of the hairpin. The third mutant protein

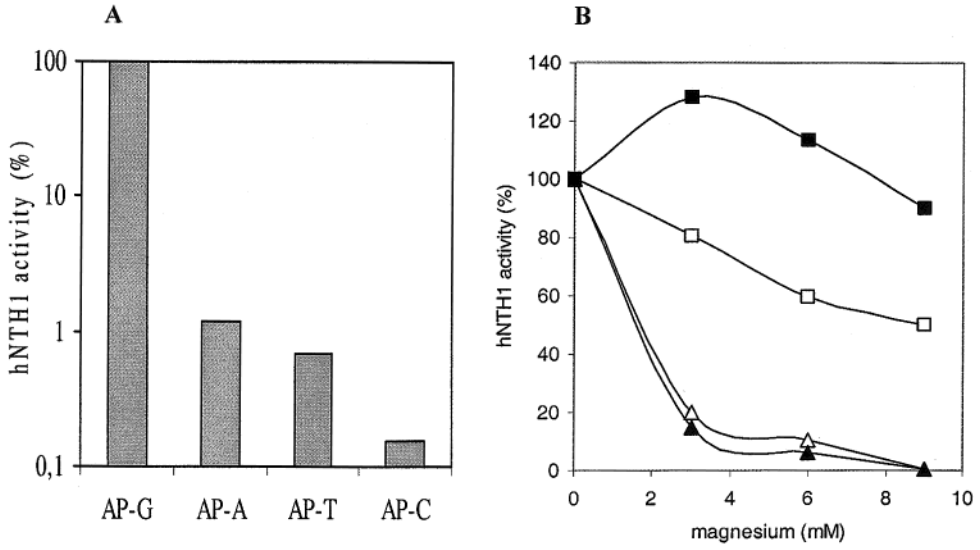


FIGURE 2: Incision of AP-DNA by hNTH1. 0.1 nM hNTH1 was incubated with 0.7 nM $^{32}$P-end-labeled AP-DNA duplexes with different bases positioned opposite the AP site in reaction buffer containing 10 mM $MgCl_2$ (A). In (B), AP-G/A from panel A (filled symbols) and AP-G/A duplexes with another sequence (open symbols) where incubated with hNTH1 in reaction buffer containing different concentrations of $MgCl_2$. Symbols: □/■: AP-G; △/▲: AP-A. The reaction mixtures were incubated for 30 min at 37 °C prior to separation by denaturing PAGE and PhosphoImaging analysis. The results are representative of three independent experiments.

Table 1: Kinetics of 5-Hydroxycytosine Incision by hNTH1 and HhH Mutants[a]

| substrate/ buffer | $K_m$ (nM) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ (rel to hNTH1 on 5ohC-G/Mg) |
|---|---|---|---|
| 5ohC-G/Mg | | | |
| hNTH1 | 0.05 ± 0.02 | 0.05 ± 0.02 | 1 |
| P211R | 0.048 ± 0.003 | 0.03 ± 0.015 | 0.8 ± 0.5 |
| +G212 | 1.2 ± 0.2 | 0.014 ± 0.008 | 0.02 ± 0.01 |
| 5ohC-A/Mg | | | |
| hNTH1 | 0.2 ± 0.1 | 0.02 ± 0.01 | 0.07 ± 0.06 |
| P211R | 0.14 ± 0.04 | 0.006 ± 0.006 | 0.02 ± 0.02 |
| +G212 | 1.7 ± 0.5 | 0.0021 ± 0.0007 | 0.0008 ± 0.0007 |
| 5ohC-G/EDTA | | | |
| hNTH1 | 0.05 ± 0.02 | 0.040 ± 0.006 | 0.9 ± 0.07 |
| P211R | 0.09 ± 0.04 | 0.035 ± 0.007 | 0.6 ± 0.3 |
| +G212 | 1.0 ± 0.3 | 0.0070 ± 0.0009 | 0.007 ± 0.003 |
| 5ohC-A/EDTA | | | |
| hNTH1 | 0.07 ± 0.03 | 0.03 ± 0.02 | 0.5 ± 0.3 |
| P211R | 0.20 ± 0.16 | 0.03 ± 0.02 | 0.2 ± 0.1 |
| +G212 | 3.5 ± 0.7 | 0.004 ± 0.002 | 0.002 ± 0.001 |

[a] Standard deviations are calculated from a minimum of three independent measurements. The $K_{cat}/K_m$ values are calculated independently.

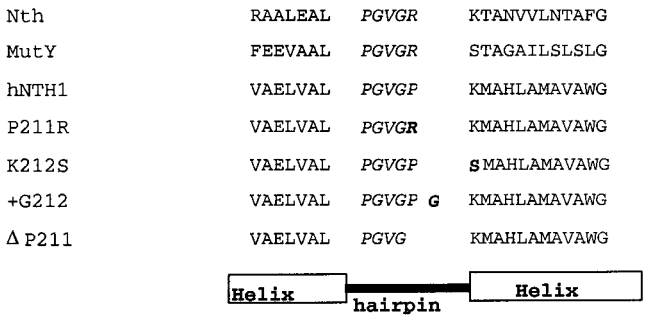


FIGURE 3: Sequences of the hNTH1 HhH mutants generated. The sequences of Nth and MutY from *E. coli* and wild-type hNTH1 are included for comparison.

was designed such that the hairpin more closely mimicked the hairpin region of the *E. coli* protein (P211R). The last mutation was introduced to resemble the HhH motif of the *E. coli* MutY protein (K212S). All mutant proteins were purified from *E. coli* AB1157 as glutathione fusion proteins and were subsequently cleaved by thrombin to isolate the free proteins. The protein purity was assessed by SDS−PAGE to be more than 90% (data not shown). The enzymatic activity of the wild-type and mutant proteins was measured by faPy DNA glycosylase assays (Figure 4). Disturbing the

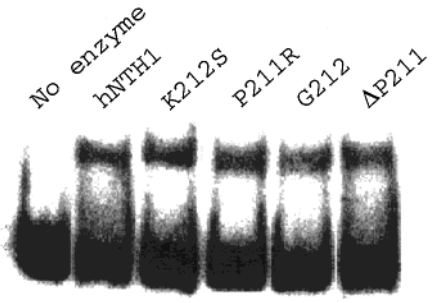


FIGURE 5: Binding of hNTH1 and HhH mutants to 5ohC-G DNA. Equal amounts of proteins were incubated with 5ohC-containing DNA and subjected to gel electrophoresis as described in Materials and Methods.

Table 2: Specific Binding of HhH Mutants to 5ohC-A/G Duplex[a]

| substrate protein | 5ohC-G | 5ohC-A |
|---|---|---|
| hNTH1 | 1.0 | 0.1 |
| K212S | 1.0 | |
| P211R | 0.11 | 0.05 |
| +G212 | 0.08 | not detected |
| ΔP211 | 0.01 | |

[a] Calculated relative to hNTH1

length of the hairpin had a dramatic effect on the faPy DNA glycosylase activity as the ΔP211 mutant protein had no activity (data not shown) while the +G212 mutant protein exhibited a 10-fold reduction. The DNA glycosylase activity of the K212S mutant protein was completely abolished (data not shown) while the P211R had an activity close to wild type.

The binding affinity of the purified proteins for a 5ohC-G base pair located in a 40-mer oligonucleotide was tested in the presence of magnesium. The labeled probe was incubated with purified proteins in the absence of competitor DNA and subjected to electrophoresis. The incubation was performed on ice, and electrophoresis was carried out at 4 °C to avoid base excision and incision of the duplex. Wild-type hNTH1 and all four mutant proteins bound the substrate (Figure 5). The specific binding constant of 5ohC-G for all proteins were determined as described (*31*), and calculated relative to the native hNTH1 in Table 2. The K212S mutant bound to DNA as strongly as the wild type, while the mutant with a shorter hairpin region, ΔP211, exhibited very low specific binding. The more than 10-fold reduction in binding specificity of the +G212 mutant correlated well with its reduced faPy excision activity (Figure 4). The binding specificity of the

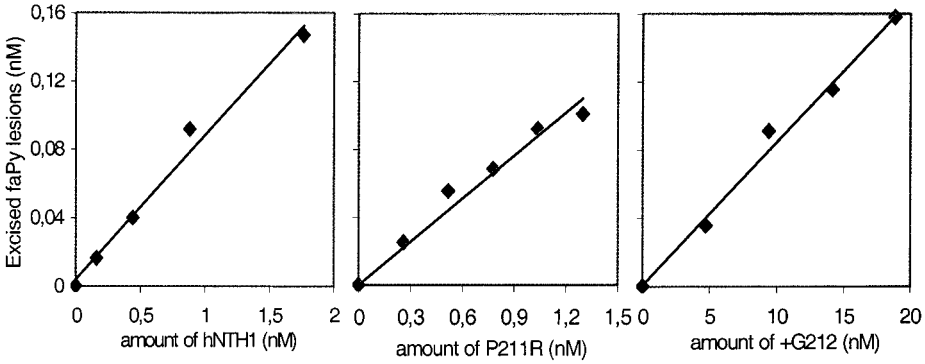


FIGURE 4: Excision of faPy lesions by native hNTH1 and mutants P211R and +G212. The mutant protein was incubated with $^3$H-faPy containing DNA as described in Materials and Methods. The figure represents the average values of four parallels obtained from two independent experiments.

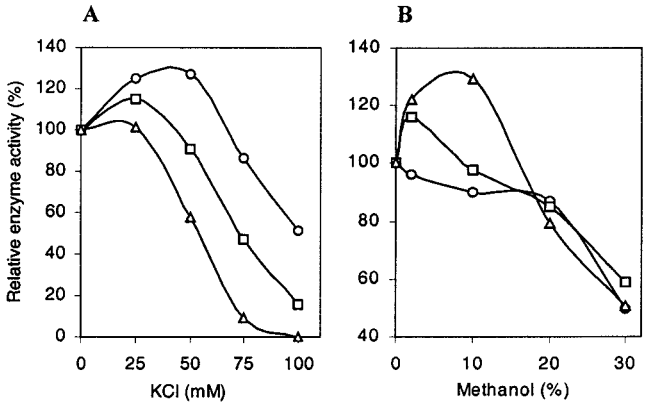


FIGURE 6: The effect of KCl and methanol on the faPy DNA glycosylase activity of the different mutant forms of hNTH1. Amounts of WT (○), PR (□), and +G (△) proteins that excise 0.1 nM of faPy residues under standard conditions were incubated with increasing KCl (A) or methanol (B). Results shown are the average of two parallels from two independent experiments.

P211R mutant was found to be lower than expected based on the faPy excision activity.

To test if the mutations interfered with the pairing base dependency for the catalytically active mutants, P211R and +G212, the kinetics for removal of 5ohC opposite adenine and guanine in different buffers was determined. The data presented in Table 1 show that to varying degrees, the mutants repaired 5ohC less efficiently than native hNTH1 as judged by the $k_{cat}/K_m$ relation. Furthermore, the detailed values for $k_{cat}$ and $K_m$ show that adenine-pairing 5-hydroxycytosine resulted in overall higher $K_m$ and lower $k_{cat}$ values for the mutant proteins (Table 1).

The specific binding constant of wild-type hNTH1 to 5ohC-A was found to be 10% of the binding to 5ohC-G (Table 2), which correlates well with the kinetic parameters. The P211R mutant bound even weaker, and no binding could be detected by the +G212 mutant even in the absence of competitor. This is in contrast to what was observed for 5ohC-G (Figure 5), where all mutants bound in the absence of competitor, and probably reflects the inability of the mutants to bind to the 5ohC-A substrate.

*Effect of Altering the Hydrophilicity of the Reaction Buffer.* Protein−DNA complex formation may depend on several factors such as ionic strength of the reaction buffer and hydrophobic interaction between the protein and the DNA bases. To visualize some of the factors important for the hNTH1−DNA interaction, wild-type protein, P211R, and +G212 were incubated with faPy-containing DNA under different ionic or hydrophobic strengths (Figure 6A and B, respectively). In accordance with data from a previous report (*9*), the wild-type hNTH1 was slightly stimulated by physiological KCl concentrations and inhibited at concentrations above 75 mM (Figure 6A). P211R behaved like wild-type hNTH1 but the +G212 mutant was inhibited, and displayed no detectable activity above 75 mM KCl. Addition of small amounts of methanol (up to 10%) to the reaction did not strongly affect the wild-type protein, whereas faPy excision by P211R and especially +G212 was stimulated. Hence, factors that lower the hydrophobic and increase the electrostatic interactions in the reaction favor the mutant proteins compared to the wild-type protein activity.

*The Human AP Endonuclease Ape1 Increases 5-ohC-A Incision by hNTH1.* To test whether the human AP endonuclease protein, Ape1, could stimulate hNTH1, the enzymes were coincubated in reactions containing 5ohC-G or 5ohC-A duplexes (Figure 7A and B, respectively). Since optimal activity of the Ape1 endonuclease requires magnesium concentrations above 4 mM (*32*), 10 mM $MgCl_2$ was used in this study. 5ohC-G was cleaved most efficiently by hNTH1 alone (Figure 7A). Incubation with Ape1 alone gave the number of AP-sites formed from spontaneously released 5ohC, which was less than 3%. Coincubation with the Ape1 protein or another AP endonuclease from *E. coli*, Nfo, did not stimulate the hNTH1 activity. Hence, hNTH1 activity upon 5ohC-G is not affected by AP endonuclease. When 5ohC-A was used as substrate (Figure 7B), addition of Ape1 stimulated incision by hNTH1 approximately 2-fold. In contrast, addition of Nfo did not produce such an effect when the total number of incisions was corrected for the amount of AP sites in the substrate (Ape1 alone). Thus, Ape1 specifically increased incision of 5ohC-A by hNTH1.

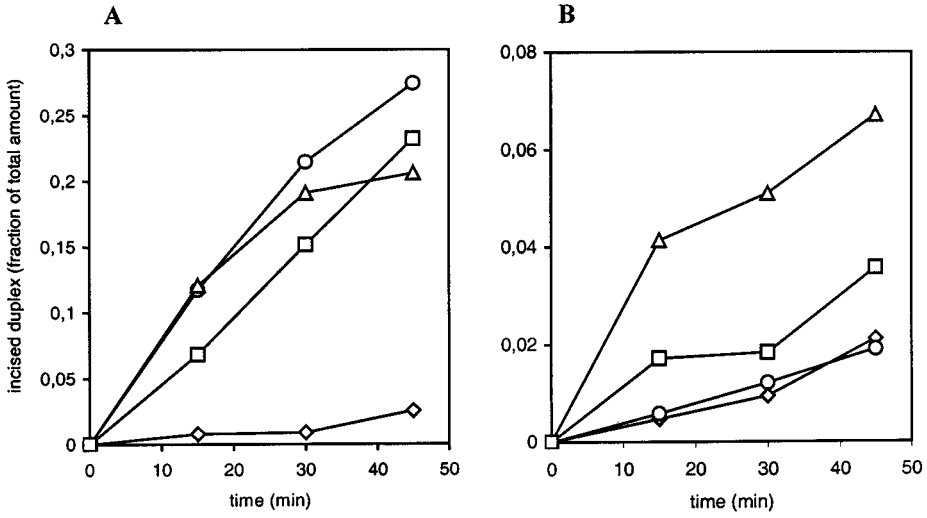


FIGURE 7: Incision of 5ohC-DNA by hNTH1 in the presence of AP endonuclease. 0.3 nM hNTH1 was incubated with 1.5 nM $^{32}$P-end-labeled 5ohC-G or 5ohC-A duplexes (A and B, respectively) at 37 °C in reaction buffer containing 10 mM $MgCl_2$. When indicated, 1 nM of Nfo or Ape1 protein was added to the mixture prior to hNTH1. The reactions are hNTH1 (○), Ape (◊), Ape with hNTH1 (△), and Nfo with hNTH1 (□). The reaction was stopped by formamide and products separated on UREA-PAGE (10%). The gels were subjected to PhosphoImaging. The points are the average of three independent experiments.

## DISCUSSION

We have studied the opposite base dependency of hNTH1 under different in vitro conditions and the role of the HhH domain in such discrimination. Activity of hNTH1 on 5ohC and abasic sites is most efficient when guanine is the opposite base. The opposite base discrimination is strongly dependent on magnesium. Furthermore, we have generated mutants for the HhH domain. Alterations in the hairpin region resulted in up to 50-fold reduction in the ability to recognize substrate; however, the mutants retained the base discrimination. Hence, the HhH domain is probably not by itself responsible for the opposite base dependent repair by hNTH1. In the absence of magnesium, Nth was reported to remove 5ohC opposite G twice as efficiently as opposite A (*5*), similar to our findings for hNTH1. The more than 10-fold better excision of faPy lesions opposite G as compared to C by Nth (*26*), implies that discriminative repair depends on the type of lesion itself, or alternatively, that the difference is due to substrate sequence variations. The biological significance of the identity of the base opposite the lesion to be excised is important to prevent a fixation of a mutation.

There is a growing list of DNA glycosylases that demonstrate opposite base dependent incision. To our knowledge, this is the first report of magnesium being important in such discrimination. The intracellular level of magnesium has been reported to reach levels of 30 mM (*33*). Hence, we believe that hNTH1 performs opposite base dependent repair in vivo. In view of hNTH1's preference for G-pairing contexts in the presence of magnesium, the inhibitory effect of magnesium reported by Ikeda and co-workers (*9*) could be related to the nature of the pairing base in the substrate used (thymine glycol). Similarly, we found that removal of faPy residues that are placed opposite cytosines was inhibited by magnesium (data not shown).

The estimated kinetic parameters for 5-hydroxycytosine differ from the reported values for dihydrouracil, especially the $K_m$ values (*9*). We do not know the reason for this apparent discrepancy, except for the differences in incubation procedure (5 versus 20 min in our experiments) and the fact that we have carrier protein in our reactions. The 10-fold difference in $k_{cat}$ values could perhaps partly be explained by some inactive proteins in our preparations, which has not been corrected for. The more than 10-fold better incision of 5ohC-G versus 5ohC-A (Table 1) does not fully agree with the difference observed at 2 mM $MgCl_2$ in Figure 1. We believe that EDTA traces from the substrate preparation might reduce the effective magnesium concentration of the results shown in Figure 1, where relatively high amounts of substrates were used.

The hairpin region is important for proper interaction with the substrate, as shown by the bandshift studies and the $K_m$ values for the incision of 5ohC by the mutants. However, the mutants display the same opposite base criteria as the wild-type protein. Therefore, it is unlikely that the HhH structure is directly involved in the identification of the opposite base. Magnesium may induce conformational changes in the DNA, hNTH1, or the complex, such that removal of an adenine-pairing lesion is disfavored. The AP−DNA incision study shows that guanine is the optimal pairing base, and indicate that hNTH1, like hOGG1, recognizes the opposite base by some direct mechanism. The crystal structure of hOGG1 suggests that the enzyme interacts with cytosine using a separate helical domain (*34*). This helical domain shares little sequence homology to hNTH1. Shekhtman et al. (*35*) reported the presence of another pseudoHhH DNA interacting domain in endonuclease III homologues. Previously, this domain has been identified in MutY as a motif that approaches the DNA backbone from the opposite side of the DNA (*36*).

The salt stimulation of hNTH1 could be explained by a more optimal conformation of the substrate or the protein. +G212 was not stimulated by salt, but on the contrary, by a hydrophobic agent. The glycosylase-active proteins display identical heat denaturation profiles, which indicate that the global conformation is not changed by the mutations (data not shown). Hence the increase in activity of +G212 in methanol might be due to improved substrate recognition under these conditions. The effect of salt/methanol can be summarized such that the activity of +G212 (and to lesser extent P211R) is stimulated by factors that enhance the hydrophilic/hydrophobic interaction relation.

The presence of Ape1 protein was found to counteract to some extent the opposite base dependent activity of hNTH1. This could not be due to AP sites created by possible noncoupled DNA glycosylase and AP lyase activity by hNTH1, since incubation with Nfo failed to increase significantly the amount of incised substrate DNA. Similarly, the faPy DNA glycosylase activity was found to be approximately 2-fold stimulated by Ape1 (data not shown). One reason the cell may want to reduce the G-specific dependency would be to improve efficiency in the removal of lesions that do not occur in G-contexts, such as thymine glycol and faPy residues. Nfo could not improve the incision by hNTH1 per se, indicating that the stimulation is specific to the human protein rather than to AP endonuclease function.

Structural studies of hOGG1 have provided insights into the discrimination mechanism. For this enzyme, a specific R residue outside of the HhH domain ($R^{154}$) has an important function in interacting with the cytosine-pairing 8-oxoguanine (*34*). The critical role of magnesium indicates that hNTH1 utilizes another mechanism for its guanine specificity. Acidic amino acid residues with potential for magnesium binding are conserved throughout the endonuclease III proteins (*8*); however, it remains to be tested whether other members of this protein group exhibit discriminative repair. Information on the three-dimensional structure of hNTH1 complexed with substrate DNA will be needed to understand the structural basis for the opposite base dependency.

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