Purification and Characterization of Human NTH1, a Homolog of *Escherichia coli* Endonuclease III

DIRECT IDENTIFICATION OF LYS-212 AS THE ACTIVE NUCLEOPHILIC RESIDUE

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The human endonuclease III (hNTH1), a homolog of the *Escherichia coli* enzyme (Nth), is a DNA glycosylase with abasic (apurinic/apyrimidinic (AP)) lyase activity and specifically cleaves oxidatively damaged pyrimidines in DNA. Its cDNA was cloned, and the full-length enzyme (304 amino acid residues) was expressed as a glutathione S-transferase fusion polypeptide in *E. coli*. Purified wild-type protein with two additional amino acid residues and a truncated protein with deletion of 22 residues at the NH₂ terminus were equally active and had absorbance maxima at 280 and 410 nm, the latter due to the presence of a [4Fe-4S] cluster, as in *E. coli* Nth. The enzyme cleaved thymine glycol-containing form I plasmid DNA and a dihydrouracil (DHU)-containing oligonucleotide duplex. The protein had a molar extinction coefficient of 5.0 × 10⁴ and a pI of 10. With the DHU-containing oligonucleotide duplex as substrate, the *Kₘ* was 47 nm, and the *kₗₘₜₖₖₜₖₖₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕₕ₅.

Reactive oxygen species are generated as by-products of oxidative phosphorylation or by ionizing radiation and induce extensive base damage that is mainly repaired via the base excision repair (BER) pathway. This repair is initiated by removal of the damaged base, catalyzed by a DNA glycosylase (1–3). There are two classes of DNA glycosylases with distinct substrate specificities: the monofunctional simple glycosylase and the glycosylase with associated AP lyase activity. All oxidized base lesions are removed from DNA by DNA glycosylase/AP lyases, which not only catalyze removal of the base lesion but also cause strand cleavage via β-elimination. The *Escherichia coli* endonuclease III (Nth) recognizes a wide range of oxidized pyrimidine derivatives, including ring-saturated and ring-fragmented derivatives such as thymine glycol (Tg), 5-hydroxycytosine, 5,6-dihydrouracil (DHU), and urea (1, 3–6). This enzyme has been well conserved from *E. coli* to the humans (7, 8). On the other hand, oxidized purine lesions are also repaired by DNA glycosylase/AP lyases, i.e. Mut M (Fpg) of *E. coli* or OGG of eukaryotes (yeast and mammals), which do not share extensive sequence similarity. Furthermore, unlike these enzymes, the 23.4-kDa *E. coli* Nth contains a [4Fe-4S] cluster (9). Structural analysis of Nth by x-ray crystallography reveals that this repair enzyme consists of two α-helical domains, which contain a helix-hairpin-helix motif and a [Fe-S]Cluster loop (10, 11). DNA binds to the cleft between these domains of the enzyme, while the catalytically important lysine and aspartic acid residues (Lys-120 and Asp-138) are positioned at the mouth of the pocket. A unified mechanism of DNA glycosylase/AP lyase activity proposed by Dodson et al. (12) suggests that Asp-138 of the protein deprotonates the Lys-120 residue, which then attacks the deoxyribose C-1 of the lesion, causing release of the base and formation of a covalent Schiff intermediate with DNA (11–13). The Schiff base intermediate undergoes several transformations resulting in strand cleavage via β-elimination (or successively δ-elimination) to leave 5′-phosphate and 3′-α,β-unsaturated aldehyde (or 3′-phosphate for βδ-elimination) ends (14). Several eukaryotic homologs of Nth have been cloned from Saccharomyces cerevisiae (15, 16), Schizosaccharomyces pombe (17), Caenorhabditis elegans (7, 8, 18),

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** The abbreviations used are: BER, base excision repair; AP, apurinic/apyrimidinic; DHU, dihydrouracil; DTT, dithiothreitol; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; Nth, endonuclease III; hNTH, human endonuclease III; PAGE, polyacrylamide gel electrophoresis; Tg, thymine glycol; ADA, N-(2-acetamido)-2-iminodiacetic acid; TAPS, 3-(1-triethylammonium)-methylpropanesulfonic acid; AMPSO, 3-(1,1-dimethyl-2-hydroxyethyl)aminolino-2-hydroxypropanesulfonic acid; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; FITC, fluorescein isothiocyanate.
and mice. Two DNA-binding motifs, as well as Lys and Asp residues corresponding to the catalytic Lys-120 and Asp-138 of *E. coli* enzyme, were well conserved among these homologs, except that *S. cerevisiae* Ntg1 does not have a [4Fe-4S] cluster (15).

Although the primary steps of the BER pathway are common to all organisms, the repair pathway in eukaryotes appears to have evolved under low stringency conditions described previously (23). One positive clone was obtained from 1 × 10⁹ independent plaques. The DNA was subcloned into pUC19 (designated as phNTH1) and the sequence was determined.

**Construction of Expression Plasmid of Glutathione S-Transferase (GST)-hNTH1 Fusion Protein**—The DNA sequence encoding amino acids 1–304 of the open reading frame was amplified by polymerase chain reaction (20 cycles at 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min) from the phNTH1 plasmid using Pfu DNA polymerase (Stratagene) and the following primers: Pmet1 (5’-CTT GGA TCC ACC GCC TGT AGC CGG AGG-3’) and Pter (5’-CTG ACA TTC AGA GAC CCT GGG CGG CCG G-3’). The polymerase chain reaction product was subcloned into the pGEX-2T plasmid at BamHI/EcoRI sites. The recombinant plasmid was designated pGEX-hNTH1met1 and confirmed the original DNA sequence.

**Expression and Purification of hNTH1 Protein**—*E. coli* BL21(D3E) plasmid carrying pGEX-hNTH1met1 was grown at 37 °C until the absorbance at 600 nm reached 0.6. The culture was cooled to 28 °C and, after the addition of IPTG to 0.1 mM, was grown at 28 °C for 4 h, prior to chilling to 0 °C. The subsequent procedures were carried out at 4 °C. After the bacteria were harvested by centrifugation, they were resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA, and 0.02% Triton X-100) and then sonicated (6 × 30 s) on ice at full power using a Braun-Sonic U. After centrifugation of the cell lysate (30 min at 15,000 × g), the supernatant was applied to a glutathione-Sepharose 4B (Amersham Pharmacia Biotech) column (5 ml) equilibrated with buffer A. After washing with buffer A, the GST fusion protein was eluted with buffer A containing 15 mM reduced glutathione (Sigma). The fractions that were a yellowish brown color were pooled and dialyzed against buffer B (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2 mM EDTA, 0.02% Triton X-100, and 6 mM 2-mercaptoethanol).

The GST-hNTH1 fusion protein was then digested with thrombin (0.28 units/mg fusion protein; Novagen) at 4 °C for 16 h to cleave the GST-ADCT. After the reaction was stopped with phenylmethylsulfonyl fluoride (1 mM), the digest was passed through a glutathione-Sepharose column equilibrated with buffer C (20 mM Tris-HCl, pH 8.0, 6 mM 2-mercaptoethanol, and 10% glycerol) containing 300 mM NaCl, to remove GST and undigested fusion protein. The GST-hNTH1 in the pooled flow-through fraction was then fractionated on an SP-Sepharose Fast Flow (Amersham Pharmacia Biotech) column with a linear gradient from 300 to 600 mM NaCl in buffer C. The fractions with the yellowish brown color were analyzed by SDS-PAGE. The full-length hNTH1 was eluted from the column later than the truncated hNTH1; these were dialyzed separately versus buffer D (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2 mM DTT, 0.005% Triton X-100, and 50% glycerol) and stored at −20 °C.

**Characterization of Human Endonuclease III**

Oligonucleotide Substrates—A DHU-containing 55-mer oligonucleotide (DHU-55) with the sequence 5’-ATT ATG CTG AGT GAT ATC CCT CTG GCC TTC GAA CCC XAC CTC AAC CTC TTC TCG CCA C-3’ (where X represents DHU) was a gift from Dr. P. Doetsch (Emory University). A uracil (U)-containing 15-mer oligonucleotide (U-15) (5’-GAG CAA UCA GCC G-3’) was purchased from Life Technologies containing 50-mer (U-50) with the sequence 5’-TCG AGG ATC CTG AGC TGC AGT CGA CGU TCG ATG ACC CGG ATC CAA GC-3’ and its complementary strand were synthesized by the NIEHS Center Core Lab at the University of Texas Medical Branch (UTMB). The oligonucleotides were gel-purified and labeled either at the 5’-end using T4 polynucleotide kinase and [γ-32P]ATP, or at the 3’-end with Klenow DNA polymerase and [α-32P]dCTP as described previously (24, 25). An oligonucleotide with a reduced AP site was prepared from a uracil-containing oligonucleotide with uracil DNA glycosylase and NaBH₄. Briefly, uracil-containing DNA (50 pmol) was incubated with 2 units of uracil DNA glycosylase (Life Technologies) in 14 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mg/ml bovine serum albumin, and 0.1 M NaBH₄ at 37 °C for 30 min. After extraction with phenol/chloroform and desalting by gel filtration the DNA was lopolysylated by vacuum centrifugation.

**DNA Glycosylase/IAP Lyase Assay**—End-labeled oligonucleotide substrates were incubated with appropriate amounts of the hNTH1 at 37 °C for 5 min in a reaction mixture (10 μl) containing 50 mM Tris-HCl, pH 8.0, 75 mM NaCl, and 1 mM DTT. After the reaction was terminated with 100 μl of 0.1% SDS, the DNA was extracted with phenol/chloroform and ethanol-precipitated along with 20 μg of glycerogen (Boehringer Mannheim). The precipitate was dissolved in 10 μl of loading solution (96% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol), heated at 95 °C for 5 min, and then loaded onto a denaturing 20% polyacrylamide gel in 7 M urea and TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). Radioactivity in the incised oligonucleotide was quantified by exposing the gel to a PhosphorImager (Molecular Dynamics). Another assay for DNA glycosylase/IAP lyase, based on the rate of conversion of damaged form I DNA to form II, was also carried out using the Tg-containing pBluescript II SK−, which was prepared by treating the DNA with 0.04% OsO₄ at 70 °C for 8 min (28). The reaction mixture (20 μl) contained 50 mM Tris-HCl, pH 8.0, 75 mM NaCl, 1 mM DTT, 250 ng of damaged plasmid DNA, and an appropriate amount of the enzyme. After incubation for 5 min at 37 °C, 2.2 μl of stopping solution (40% glycerol, 0.2% bromphenol blue, and 1% SDS) was added, and the samples were electrophoresed on 0.8% agarose gels in TBE buffer. The intensities of the bands of form I and form II DNA were quantitated from an image of the gel captured using an Epson flat bed scanning video system and attached software (Stratagene). The average number of nicks (or Tg) per molecule was calculated by assuming a Poisson distribution of Tg formation in the plasmid (27).

**Tg-DNA Glycosylase Assay**—Plasmid pBluescript II SK− DNA was labeled with [methyl-3H]thymidine 5′-triphosphate by the random priming method and treated with 0.4% OsO₄ at 85 °C for 10 min. A reaction mixture (20 μl) containing 50 mM Tris-HCl, pH 8.0, 75 mM NaCl, 1 mM DTT, and 50 μg of OSO₄-treated DNA (2.9 × 10⁵ cpm) was incubated at 37 °C for 30 min with different concentrations of purified proteins. After incubation, 30 μl of stopping solution (0.5 mM sodium acetate and 0.67 mg/ml of calf thymus DNA) and 125 μl of cold ethanol were added. The DNA was precipitated at −80 °C and centrifuged at 15,000 × g for 15 min, and the ethanol-soluble radioactive material was measured in supernatant by scintillation counting.

**DNA Trapping Assay with OsCNBH₄**—The DNA-trapping reaction was performed in a 10-μl mixture containing appropriate amounts of hNTH1 and substrate duplex oligonucleotide, 50 μM Tris-HCl, pH 8.0, 1 mM EDTA, and 50 μM OsCNBH₄ (Aldrich). After incubation at 37 °C for 10 min, the samples were boiled for 3 min in standard SDS-PAGE loading buffer and separated by electrophoresis on a 12.5% SDS-polyacrylamide gel (28). The gel was dried on DE81 paper (Whatman) and analyzed by autoradiography. Relative trapping efficiencies were determined by PhosphorImager analysis.

**Electrophoretic Mobility Shift Assay**—Each sample (10 μl) contained 10 mM [32P]-labeled probe DNA, 4 mM GST-hNTH1 fusion protein, 50 mM Tris-HCl (pH 8.0), 75 mM NaCl, 20% glycerol, and 1 μg of poly(dI-dC)/poly(dI-dC) (poly(dI-dC)). The sample was incubated at 25 °C for 30 min prior to loading onto a 15% non-denaturing polyacrylamide gel containing 0.5 × TBE. The gels were run at 4 °C for 3 h, dried, and analyzed by autoradiography.

Identification of the Amino Acid Residue in hNTH1 That Forms Covalent Linkage to the Substrate—To make AP site-containing DNA, a [32P]-labeled U-13 oligonucleotide duplex (42 nmol) was incubated with 175 units of uracil DNA glycosylase in a reaction buffer containing 50
mm Tris·HCl (pH 8.0) and 1 mm EDTA. After the reaction at 37 °C for 30 min, hNTH1 (about 60 nmol) was allowed to react with the AP site-containing DNA in the presence of 50 mm NaCNBH₃ at 37 °C for 2 h. After the reaction mixture was loaded onto a 5-ml Econo-Pac Q cartridge (Bio-Rad) equilibrated with 20 mM Tris·HCl (pH 9.0), it was washed with equilibration buffer, and the trapped protein-DNA complex was eluted with a linear gradient of 0–0.5 M NaCl in 20 mM Tris·HCl (pH 8.0). The fractions were assayed by SDS-PAGE and Coomasie Blue staining for the presence of the complex. The fractions from 0.15 to 0.25 M NaCl containing the complex (12 nmol) were pooled and dialyzed versus 50 mM NH₄HCO₃. After boiling for 5 min, the complex was hydrolyzed with a combination of 20 μg of endoproteinase Glu-C and 20 μg of trypsin (Promega, sequence grade) at 37 °C for 24 h, and the treatment was repeated for an additional 48 h. The digest was lyophilized in a Speed Vac, dissolved in 0.1% trifluoroacetic acid, and applied to a Vydac C18 (300-Å pore, 5-mm diameter) reversed-phase HPLC column (1 x 250 mm) equilibrated with 15% acetonitrile containing 0.1% trifluoroacetic acid. The column was eluted at a flow rate of 80 μl/min with a gradient (35 min) of 15–40% acetonitrile containing 0.1% trifluoroacetic acid.

Construction of Site-specific Mutants of hNTH1—The mutants K212Q and K212R were generated using Stratagene's Chameleon dou- foot printing was performed essentially as described previously (25). Elmer/Applied Biosystems Procise protein/peptide sequencer. DNase I containing 0.1% trifluoroacetic acid. The column was eluted at a flow rate of 80 μl/min with a gradient (35 min) of 15–40% acetonitrile containing 0.1% trifluoroacetic acid. The column was eluted at a flow rate of 80 μl/min with a gradient (35 min) of 15–40% acetonitrile containing 0.1% trifluoroacetic acid.

RESULTS

Overexpression of Recombinant hNTH1 in E. coli and Purification of the Enzyme—The sequence of the hNTH1 cDNA independently cloned and used in this study is identical to the published sequence of Hilbert et al. (7), except that our clone has two additional nucleotides at the 5'-end. The sequence of another hNTH1 cDNA cloned by Aspinwall et al. (8) is different from ours by three nucleotide substitutions within the coding region, as well as a 33-nucleotide addition at the 5'-end. The number of amino acid residues of hNTH1 cited here is based on our sequence and that of Hilbert et al. (7).

The DNA sequence encoding amino acids (1–304) of the hNTH1 was amplified by polymerase chain reaction, and the resulting product was inserted into a pGEX-2T vector to over-express the full-length hNTH1 fused to the C terminus of the GST protein. SDS-PAGE of the proteins from bacteria induced or uninduced by IPTG indicated that the 60-kDa protein was overexpressed from the plasmid (data not shown); this molecular mass agreed well with the expected size (59.8 kDa) of the GST-hNTH1 fusion protein. We used proteinase-deficient E. coli to overexpress the enzyme. The majority of the fusion protein was expressed in the soluble form by incubating the cells at 28 °C after the addition of IPTG and was readily purified by affinity chromatography (Fig. LA).

The GST-hNTH1 fusion protein was subsequently cleaved by thrombin to isolate the wild-type hNTH1. We observed two polypeptides of 36 and 34 kDa on SDS-PAGE in the thrombin digest in addition to the 26-kDa band (GST), indicating that thrombin cleaved at two sites in the fusion protein (Fig. LB). NH₂-terminal amino acid sequencing of product b showed that the protein was hNTH1 with two additional amino-terminal amino acids (Gly-Ser) derived from the vector. The NH₂-termi-
nal sequence analysis of band c indicated that this band was a truncated form of hNTH1 lacking 22 residues at the NH₂ terminus. The conditions for thrombin digestion were optimized for maximum generation of the full-length protein. After removal of uncleaved protein and GST by chromatography on a glutathione affinity column, two hNTH1 polypeptides with different sizes were finally separated from each other by Sepharose chromatography, and each was judged to be apparently homogeneous by SDS-PAGE (Fig. 1C, lanes 5 and 6).

Some physicochemical properties of the full-length hNTH1 protein are summarized in Table I. The values of pl and E₄₁₀/E₂₈₀ were determined experimentally and are in excellent agreement with the calculated values. The GST-hNTH1 fusion protein and the purified full-length and truncated hNTH1 proteins are all yellowish brown and have an absorption maximum at 410 nm (data not shown).

**DNA Glycosylase/AP Lyase Activity of Purified hNTH1—**

The activity of the purified hNTH1 protein was measured using a Tg-containing plasmid DNA and a DHU-containing oligonucleotide. Full-length and truncated hNTH1 nicked the form I Tg plasmid, but not the normal plasmid, at about the same rate to convert it into form II (Fig. 2A). The GST-hNTH1 fusion protein also had Tg-DNA-specific nicking activity, as previously reported by Hilbert et al. (7), but the specific activity of the protein was about half that of the non-fusion enzymes. The hNTH1 incised the DHU-containing oligonucleotide at the DHU site to generate a 3'-α,β-unsaturated aldehyde (Fig. 2B, lane 2), which could subsequently be converted to a 3'-OH terminus by treatment with E. coli endonuclease IV (Fig. 2B, lane 3). This showed that hNTH1 had an AP lyase activity that cleaved damaged DNA via β-elimination. The product of β-elimination, with a 3'-phosphate terminus, was not observed.

The pH optimum of hNTH1 was established to be pH 8 with the Tg-containing plasmid substrate (Fig. 3A). The maximal activity of hNTH1 was observed in 75 mM NaCl or KCl, and higher salt concentrations inhibited the enzyme (Fig. 3B). EDTA was not inhibitory even at 10 mM concentration (Fig. 3C). Some divalent cations (Mg²⁺, Mn²⁺, and Ca²⁺) inhibited hNTH1, and about 50% of the normal activity was observed in the presence of 5–10 mM concentrations of these ions (Fig. 3C).

We measured the reaction rate of hNTH1 with DHU-containing oligonucleotide duplexes having different bases opposite the DHU (data not shown). DHU is a derivative of cytosine, so its opposite base should be guanine, but DHU is able to pair to adenine during DNA replication. That hNTH1 cleaved both duplex oligonucleotides at nearly the same rate indicated that the enzyme activity was not significantly affected by the presence of A or G opposite DHU.

### Table I

**Physicochemical properties of recombinant hNTH1**

| Property                          | Value     |
|----------------------------------|-----------|
| Molecular mass (observed)        | 36,000    |
|                                  | (predicted) 33,548 |
| Isoelectric point (pl)           | 4.8       |
| Isoelectric focusing             | ~10       |
| Molar extinction coefficient (E₄₁₀/E₂₈₀) | 10.2     |
| Experimental                     | 5.0 × 10⁴ |
| Calculated                       | 4.8 × 10⁴ |
| E. coli Nth                      | 1.9 × 10⁴ |
| 410/280 nm absorbance ratio      | 0.23      |
| hNTH1 in this study              | 0.23      |
| hNTH1 of Aspinwall et al.        | 0.38–0.4  |
| E. coli Nth                      | 0.38–0.4  |

*The value was calculated from amino acid sequence data by the equation of Gill and von Hippel (40).*

*Data from Aspinwall et al. (6).*

*Data from Asahara et al. (41) and Thayer et al. (11).*
DNase I (Fig. 4C). When the complementary strand was labeled, protection was observed spanning a zone of 15-nucleotide with a hypersensitive site region in between (Fig. 4D). No protection was observed when unmodified oligonucleotide was used (Fig. 4A). Schematic representations of the protected region or hypersensitive sites are depicted in Fig. 5. Similar footprints were obtained using oligonucleotides containing a reduced AP site or tetrahydrofuran at the site of DHU (data not shown).

Cross-linking of the Reaction Intermediate by NaCNBH₃—DNA glycosylases with associated AP lyase activity normally use an amino group as a nucleophile, resulting in a transient covalent imino enzyme-DNA substrate Schiff base intermediate (12, 30). This intermediate can be stabilized by reduction with NaCNBH₃ and monitored by DNA band shifts on SDS-polyacrylamide gels (DNA trapping assay). The assay using a DHU-55 oligonucleotide duplex showed that hNTH1 can be trapped with the DHU-containing oligonucleotide (Fig. 6A). That a dihydro-U:A oligonucleotide was trapped with the same efficiency as the dihydro-U:G oligonucleotide (data not shown) confirmed that the reaction with hNTH1 was independent of the complementary strand. The AP site-containing oligonucleotide was also a good substrate for trapping by hNTH1 (Fig. 6B).

Quantitation of the covalent complex formed in the DNA trapping assay established the fraction of active molecules in the hNTH1 preparation. The hNTH1 protein (1 pmol) was incubated with an increasing concentration of DHU-containing oligonucleotide in the standard buffer in the presence of NaCNBH₃ (Fig. 6C). After incubation at 37°C, the reactions were analyzed for the number of hNTH1 molecule covalently trapped on DNA. The maximum complex formation (calculated by extrapolation from a double reciprocal plot of the data in Fig. 6C) was 0.25 pmol, indicating that 25% of the hNTH1 molecules were active.

Identification of the Residue That Forms Covalent Linkage to the Substrate DNA—The sequence homology of hNTH1 to E. coli Nth around the putative catalytic site suggested that the Lys-212 of hNTH1 was the nucleophilic residue that formed a covalent DNA complex. To confirm this possibility, we sequenced an HPLC-purified radiolabeled peptide complex obtained by proteolysis of a borohydride-trapped protein-DNA complex prepared using a radiolabeled 13-mer oligonucleotide substrate. After digestion of the complex with endoproteinase Glu-C and trypsin, the peptide fragment bearing the radiolabeled oligonucleotide eluted from a C18 reversed-phase HPLC column at about 30% acetonitrile (Fig. 7). The results of microsequence analysis of the HPLC fraction containing the peptide complex are shown in Table II. Two sequences, one major and one minor, were obtained. The major sequence was identical to the reported sequence of hNTH1 in the region representing Lys-212. Sequence analysis results of cycle 10 (Lys-212)
showed a blank for the major peak, consistent with a lysyl e-amino group adducted with an oligonucleotide. Highly charged 2-anilino-5-thiazolinone amino acid residue complexes formed in sequencing reactions typically are not solvent-extractable from the reaction cartridge of the sequencer and thus give a negative result at that position run during sequence analysis. The minor sequence was established to be a combination tryptic and autocatalytic peptide cleavage product of endoproteinase Glu-C representing the sequence region Thr-191 to Lys-206. Cycle 10 of the endoproteinase Glu-C peptide sequence gave the expected yield for Thr-200, which served as an internal control and demonstrated the absence of any instrument malfunction at this cycle. Therefore, we concluded that Lys-212 was covalently cross-linked to DNA in the trapped hNTH1-substrate complex.

Site-Directed Mutagenesis of a Catalytic Residue Lys-212—To further confirm the involvement of this lysine residue in the enzyme’s catalytic mechanism, Lys-212 was replaced with Gln or Arg by site-directed mutagenesis. The mutant proteins (K212Q and K212R) were expressed in a soluble form as GST fusion proteins in E. coli. The specific absorbance of each mutant protein at 410 nm was the same as that of the wild type protein, indicating that the mutant proteins each has an intact [4Fe-4S] cluster (data not shown). The K212Q mutant could not form a covalent complex with the DHU-containing oligonucleotide, and had no DNA glycosylase and AP lyase activity (Fig. 8, A–C). On the other hand, the K212R mutant could trap the DNA substrate and had DNA glycosylase/AP lyase activity, but at a lower level than the wild-type protein. Kinetic studies showed that K212R had about a 2.5-fold higher $K_m$ and a 35-fold lower $k_{cat}$ than the wild type (Table III).
Characterization of Human Endonuclease III

However, both mutant proteins formed a gel-shifted complex with a duplex containing a reduced AP site, indicating that the mutation of Lys-212 did not affect the specific DNA binding activity of hNTH1 (Fig. 8D).

**Immunological Characterization of hNTH1**—A polyclonal antibody raised against hNTH1 used in immunoblotting experiments with HeLa S3 cell extract (Fig. 9A); a single band of 36 kDa with the same molecular mass as that of the recombinant protein was detected by SDS-PAGE. This result showed that the endogenous NTH was not only identical to the cloned enzyme but also may not have extensive post-translational modification. Finally, indirect immunofluorescence studies indicated that hNTH1 was distributed in both the nucleus and the cytoplasm (Fig. 9B, b). However, in some cells, the enzyme was concentrated in the nucleus (Fig. 9B, c).

**DISCUSSION**

We have independently cloned the cDNA of human NTH and expressed the recombinant protein in *E. coli* in soluble form. Our clone is nearly identical to the hNTH1 cDNA reported by Aspinwall et al. (8). Although some preliminary properties of the enzyme were described in those earlier papers, we have provided a more comprehensive characterization of the enzyme in this report. The full-length enzyme with 304 amino acid residues was expressed as a GST fusion polypeptide whose cleavage with thrombin resulted in the production of the wild type protein with two additional NH2-terminal amino acid residues and a truncated protein with a deletion of 22 residues at the amino terminus (Fig. 1B).

**Table III**

| Enzymes | *Km* (nM) | *kcat* (min⁻¹) | *kcat/Km* (min⁻¹ nM⁻¹) |
|---------|-----------|----------------|------------------------|
| Wild type | *nw* | 69 | 0.12 | 1.7 × 10⁻³ |
| K212R | 170 | 0.0034 | 0.02 × 10⁻³ |

Kinetic constants of wild type and K212R mutant of hNTH1-GST for DHU-containing oligonucleotide

**Fig. 8. Activity of mutant hNTH1.** A, DNA trapping assay of the mutant proteins. Wild type (WT) or mutant proteins (2 pmol) were allowed to react with 32P-labeled DHU-55 oligonucleotide duplex at 37 °C for 10 min in the presence of 50 mM NaCNBH3. The reaction products were analyzed as described for Fig. 7. B, DNA glycosylase/AP lyase activities of mutant proteins. Various amounts of each enzyme were incubated with 250 ng of Tg-containing plasmid at 37 °C for 30 min, and the resulting nicks were quantified. C, Tg-DNA glycosylase activity of mutant proteins. Various amounts of each enzyme were incubated with 300 ng of 3H-labeled Tg-containing DNA at 37 °C for 30 min, and the ethanol-soluble radioactivity was measured. D, electrophoretic mobility shift assays of mutant proteins. U-13 oligonucleotide and reduced AP (rAP) site-containing oligonucleotide derived from U-13 were used as probes.

**Fig. 9. Immunological analysis.** A, Western blot detection of hNTH1 in HeLa cells. HeLa S3 cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.5% Nonidet P-40, and proteins were fractionated by SDS-PAGE (12.5% polyacrylamide). The hNTH1 band (lane 1) was visualized by affinity-purified anti-hNTH1 antibody. Lane 2, purified full-length hNTH1 (20 pg). B, intracellular localization of hNTH1 in HeLa S3 cells. Cells on coverslips were fixed and exposed to anti-hNTH1 antibody and then exposed to fluorescein isothiocyanate-labeled secondary antibody under a Zeiss UV microscope.

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*Hilbert et al.* (7) but significantly different in sequence from the second hNTH1 cDNA reported by Aspinwall et al. (8). Although some preliminary properties of the enzyme were described in those earlier papers, we have provided a more comprehensive characterization of the enzyme in this report. The full-length protein was detected by SDS-PAGE. This result showed that the endogenous enzyme in HeLa cells has the same size as a recombinant protein in SDS-PAGE by Western blotting and is apparently localized in both the nucleus and cytoplasm as indicated by an immunofluorescence assay. However, in some cells, the enzyme is predominantly present in the nucleus. Furthermore, the cytoplasmic enzyme may actually be localized in the mitochondria.

Purified hNTH1 is yellowish brown in color and has an absorbance spectrum with a peak at 280 nm and a second peak at 410 nm due to the presence of a [4Fe-4S] cluster (Table I). Four Cys residues of this domain are well conserved among eukaryotic Nth homologs (7, 8) and are suggested to form a pocket with a domain of the helix-hairpin-helix motif important.
for DNA binding (11). The absorbance ratio of 410/280 nm is useful as an index of the intactness of the [4Fe-4S] cluster (11). The value (0.23) for hNTH1 is smaller than that of E. coli Nth (0.38–0.40), which may reflect the difference in the specific absorbance of these proteins at 280 nm (Table I). Titration of the recombinant protein with an oligonucleotide substrate indicated that only about 25% of the enzyme molecules in a typical preparation were active, probably due to misfolding of the eukaryotic protein during expression in E. coli.

The recombinant human homolog of E. coli Nth has been shown earlier to be nearly identical to Nth in enzyme activity (7, 8). The enzyme releases Tg, urea, and other residues from the damaged DNA and has an AP lyase activity that cleaves (7, 8). The enzyme releases Tg, urea, and other residues from shown earlier to be nearly identical to Nth in enzyme activity the eukaryotic protein during expression in a typical preparation were active, probably due to misfolding of the eukaryotic protein during expression in E. coli.

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