Mutation at Active Site Lysine 212 to Arginine Uncouples the Glycosylase Activity from the Lyase Activity of Human Endonuclease III†

Xiang Liu‡ and Rabindra Roy*,‡

Sealy Center for Molecular Science, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555

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ABSTRACT: The human endonuclease III (hNTH1) is an important DNA glycosylase with associated abasic lyase activity. We previously demonstrated that the K212Q mutant was totally inactive, while the K212R mutant had reduced DNA glycosylase/lyase activity and could form a covalent complex with the substrate DNA upon reduction. We further characterized the biochemical properties of this K212R mutant protein. NH_2- (N-) terminal sequencing in combination with mass spectrometry of the peptide−DNA adduct suggested that "opportunistic" lysine(s) in the lysine-rich N-terminal tail formed a Schiff base which might result in β-elimination. However, simultaneous substitution of Lys-75 with Gln and deletion of first 72 residues in the N-terminal tail could not cause further alteration in the glycosylase reaction or β-elimination event. Nonetheless, the time kinetics of K212R and its subsequent mutants showed glycosylase activity without any detectable AP-lyase activity during the first 10 min of the reaction. These results suggest that a single point mutation at the active site (K212R) uncoupled the glycosylase activity from the lyase activity. We propose that the uncoupled reaction carried out by K212R is a result of direct attack either by the nonionized form of the guanidino group of arginine which forms an unstable Schiff base that hydrolyzes prior to the β-elimination event or by hydroxide ion to cleave the glycosylic bond. In either case this reaction is followed by a secondary β-elimination event performed by random lysine residues primarily from the N-terminal tail region.

Oxidative stress is arguably the most important cause of cellular genotoxicity, which is implicated in the etiology of a variety of pathophysiological disorders including aging and cancer (*1*). The reactive oxygen species (ROS)[1] are generated endogenously in cells as byproducts of oxidative phosphorylation and enzymatically during inflammatory responses and detoxification reactions. ROS are also generated exogenously due to metal toxicity, ionizing radiation, and other environmental agents and are by far the most important genotoxic agents that cause various mutagenic, carcinogenic, and cytotoxic lesions in DNA (*2*). The oxidative DNA damage includes single- and double-strand breaks and a myriad of base damage. These oxidative base lesions are generally repaired via the base excision repair (BER) pathway in bacteria and eukaryotes, initiated by removal of the damaged base by a damage-specific DNA glycosylase or glycosylase/lyase (*3*−*5*). There are two types of DNA glycosylases with distinct substrate specificities: the monofunctional simple glycosylases and the glycosylases 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- or β,δ-elimination. Among such oxidatively damaged bases, a series of toxic and mutagenic structurally diverse oxidized pyrimidines are repaired by endonuclease III (NTH), a glycosylase/lyase present in all species from bacteria through man. Following the DNA glycosylase/AP-lyase reaction, subsequent repair steps include removal of the 3′-α,β-unsaturated aldehyde by phosphodiesterase activity of AP endonuclease (APE) and filling the resulting DNA gap with a DNA polymerase and finally sealing of the repaired strand by DNA ligase (*5*, *6*).

The NTH family of repair glycosylase/lyases constitutes a conserved class of enzymes that seem to be present throughout phylogeny (*5*). The *Escherichia coli* Endo III (Nth) recognizes a wide range of oxidized pyrimidine derivatives, including ring-saturated and ring-fragmented derivatives such as thymine glycol (Tg), 5-hydroxycytosine, dihydroxyuracil (DHU), urea, and six other oxidized pyrimidines (*3*, *5*, *7*−*9*). Despite a significant amount of sequence homology, *E. coli* and eukaryotic NTHs display some diverse substrate specificity in addition to a large degree of overlap. On the other hand, oxidized purine lesions are repaired by MutM (Fpg) in *E. coli* or OGG in yeast and mammals, which are DNA glycosylase/AP lyases but have no extensive sequence similarity. Significantly, and unlike these enzymes, the 23.4 kDa *E. coli* Nth, a monomeric protein requiring no

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* Corresponding author. Telephone: 914-789-7130. Fax: 914-592-6317. E-mail: rabinroy@aol.com.

‡ Present address: Division of Carcinogenesis and Molecular Epidemiology, American Health Foundation, 1 Dana Road, Valhalla, NY 10595.

[1] Abbreviations: ROS, reactive oxygen species; BER, base excision repair; AP, apurinic/apyrimidinic; DHU, dihydrouracil; IPTG, isopropyl β-D-thiogalactopyranoside; GST, glutathione *S*-transferase; hNTH1, human endonuclease III; Nth, *E. coli* endonuclease III; PAGE, polyacrylamide gel electrophoresis; Tg, thymine glycol; HPLC, high-pressure liquid chromatography.

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cofactor, contains a [4Fe-4S] cluster (*10*). Several eukaryotic homologues of *E. coli* Nth have been cloned in *Saccharomyces cerevisiae* (*11*), *Schizosaccharomyces pombe* (*12*), *Caenorhabditis elegans* (*13*), humans (*14*, *15*), and mouse (*16*). The five DNA-binding motifs including the helix−hairpin−helix (HhH) and the [4Fe-4S] cluster, as well as Lys and Asp residues corresponding to the catalytic Lys-120 and Asp-138 of the *E. coli* enzyme, are well conserved among these homologues, except that *S. cerevisiae* NTG1 does not have a [4Fe-4S] cluster (*11*, *17*−*18*).

A unified mechanism of DNA glycosylase/AP-lyase activity proposed by Dodson et al. (*19*) suggests that Asp-138 (Asp-231 in hNTH1) of the *E. coli* Nth deprotonates the Lys-120 (Lys-212 in hNTH1) residue, which then attacks the deoxyribose at C-1′ of the lesion, causing release of the base and formation of a covalent Schiff base intermediate with DNA (*18*−*20*). 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 β,δ-elimination) ends (*21*). The monofunctional glycosylases catalyze the C1′−*N*-glycosyl bond cleavage to produce AP sites. The cleavage is mediated by a water molecule, which is activated by an acidic residue, Asp or Glu of the enzyme (*19*).

In our previous paper we found that the replacement of Lys-212, the active site nucleophile in hNTH1, with Glu completely inactivated the enzyme. Interestingly, replacement with Arg yielded an active enzyme with about 85-fold lower catalytic specificity than the wild-type (WT) protein (*14*). In this study we found that the glycosylase and lyase activities of the K212R protein are uncoupled. The random lysine residue(s) primarily from the N-terminal region is (are) responsible for the residual lyase activity in the K212R mutant enzyme.

MATERIALS AND METHODS

Expression and Purification of the Full-Length hNTH1 K212R Mutant Protein. The full-length hNTH1 cDNA carrying the K212R mutation was excised from the pGEX-2T-hNTH1 K212R plasmid (*14*) and subcloned into the pGEX-6P1 expression vector at *Bam*HI/*Eco*RI sites. The recombinant plasmid was designated pGEX-6P1-hNTH1 K212R, and the original DNA sequence including the desired mutated base was confirmed. The pGEX-6P1-hNTH1 K212R was transformed into BW nth$^-$nei$^-$ *E. coli* cells that are deficient in endonuclease III and endonulease VIII. The cells carrying pGEX-6P1-hNTH1 K212R were grown as described (*14*). The glutathione *S*-transferase (GST)−hNTH1 K212R fusion protein was purified following the procedure as described (*14*) with some modifications. The cell-free extract was allowed to mix with glutathione−Sepharose 4B (Amersham Pharmacia Biotech) beads according to the manufacturer's instructions. After being washed with buffer A (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 2 mM EDTA, and 0.02% Triton X-100), the GST fusion protein bound to the beads was digested with PreScission protease (80 units/mL GST−Sepharose; Amersham Pharmacia Biotech) at 5 °C for 4 h to cleave the GST adduct. Then the beads were eluted with buffer A containing 10 mM reduced glutathione (Sigma). The yellowish brown colored fractions were pooled and passed through a SP-Sepharose column equilibrated with buffer B (20 mM Tris-HCl, pH 8.0, 6 mM 2-mercaptoethanol, and 10% glycerol), containing 100 mM NaCl, to remove GST and undigested fusion protein. The 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 100 to 800 mM NaCl in buffer B. The fractions with the yellowish brown color were analyzed by SDS−PAGE and stored in aliquots at −80 °C.

Oligonucleotide Substrates. A DHU-containing 56-mer oligonucleotide (DHU-56) with the sequence 5′-ATT ATG CTG AGT GAT ATC CCT CTG GCC TTC GAA CCC XAC CTC AAC CTC TGC CCA CC-3′ (where X represents DHU) and a uracil- (U-) containing 13-mer oligonucleotide (U-13) (5′-GCA CAG UCA GCC G-3′) were purchased from Operon. The oligonucleotides were gel-purified and labeled at the 5′-end using T4 polynucleotide kinase and [^{32}P]ATP as described previously (*22*). The ^{32}P-labeled DHU-56 was mixed with a 1.7-fold molar excess of the complementary strand for annealing. The mixture was boiled for 1 min and cooled to room temperature in 1 h in a buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl. Finally, the labeled oligonucleotide was separated from free [^{32}P]ATP in Sephadex G-50 (Pharmacia) nick spin columns. Under these conditions the substrate preparation contained 60−65% duplex oligonucleotides as analyzed in 10% nondenaturing gel.

Purification and Sequencing of the Cross-Linked Complex of the K212R Protein to a 13-mer Duplex Oligonucleotide Containing an AP Site. The cross-linked complex was obtained by incubation of the AP site-containing DNA with hNTH1 K212R protein (90 nmol) in the presence of 50 mM $NaCNBH_3$ at 37 °C for 2 h as described (*14*). After being separated by a 5-mL Hitrap Q-Sepharose (Pharmacia) column and dialyzed versus 50 mM NH_4HCO_3, 200 μL of the protein−DNA complex was exhaustively hydrolyzed with 20 μg of endoproteinase Glu-C (Promega, sequence grade) at 37 °C for 20 h. The digest was dried under reduced pressure with a centrifugal evaporater. The dried sample (5000 dpm) was then dissolved in 0.5 mL of 0.1% trifluoroacetic acid, applied to a Jupiter C18 (Phenomenex, 300 Å pore, 5 μm particle) reversed-phase HPLC column (4.6 × 250 mm), and chromatographed as described (*14*). The radioactivity of all fractions was measured by scintillation counting. Then, every fraction containing radioactivity was divided into two aliquots and dried. One aliquot was submitted to automated N-terminal amino acid sequence analysis in the University of Texas Medical Branch (UTMB) Core Protein Chemistry Laboratory, using a Perkin-Elmer/Applied Biosystems Procise protein/peptide sequencer. The other aliquot was used to measure the mass by electrospray mass spectrometry on a VD platform (Micromass) single quadrupole mass spectrometer in Dr. Robert Fox's laboratory at UTMB. Typically, the instrument was scanned from *m*/*z* 600 to *m*/*z* 1600 in 10 s with the ion source set at 3.2 kV; 10 scans were acquired for each sample. Horse heart myoglobin, with an average mass of 16951.50 Da (Sigma), was used as the standard for instrument calibration. Samples of the protein−DNA complex at approximately 0.01 mg/mL in 50% acetonitrile and 0.1% formic acid were introduced into the spectrometer at 5 μL/min. Nitrogen at 40 mL/min was used to pneumatically assist the electrospray process.

Construction of hNTH1 Mutants. The double mutant K212R-K75Q was generated using Stratagene's Chameleon double-stranded site-directed mutagenesis kit. The primer used to mutate the *Mlu*I site of the vector was 5′-TCG CGC AAC GTA TCA GTG GGC-3′. The mutagenic primer was 5′-GAG CCC CTC CAG GTA CCA GTC-3′. The entire hNTH1 amino acid coding region was sequenced to ensure the presence of only the desired mutation.

The DNA sequence encoding hNTH1 NΔ72, hNTH1 K212R-NΔ72, or hNTH1 K212R-K75Q-NΔ72 was amplified by polymerase chain reaction (30 cycles at 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min) from pGEX-6P1-hNTH1, pGEX-6P1-hNTH1-K212R, or pGEX-6P1-hNTH1-K212R-K75Q plasmids using Perkin-Elmer PCR core reagents. The PCR products were subcloned into the pGEX-2TK plasmid at *Bam*HI/*Eco*RI sites. The recombinant plasmids were confirmed for the original DNA sequences. The purification of the mutant proteins was performed as above, except the truncated hNTH1 fusion proteins were digested by thrombin.

Enzyme Cleavage Assay. The 5′-labeled duplex oligonucleotide substrate (DHU; 15 nM) was incubated with an appropriate amount of each protein at 37 °C for different times in a reaction mixture containing 50 mM HEPES−KOH, pH 7.9, 75 mM NaCl, 0.1 mg/mL BSA, 0.5 mM EDTA, and 1 mM DTT. At each time point, two aliquots (10 μL each) were taken from the reaction mixture, and the reaction was stopped by adding 25 μL of stop solution (90% formamide, 0.025% bromphenol blue, 0.025% xylene cyanol, and 4% glycerol). Two hundred millimolar piperidine was added to one aliquot to measure the glycolyase activity. Then, all of the mixtures were heated at 95 °C for 5 min, and 5 μL of the mixture was loaded onto a denaturing 10% polyacrylamide sequencing gel in 7 M urea and TBE buffer (89 mM Tris-HCl, pH 8.0, 89 mM boric acid, and 2 mM EDTA). Radioactivity in the incised oligonucleotide was quantified by exposing the gel to a PhosphorImager (Molecular Dynamics).

Other Methods. Proteins were quantified by the dye-binding method (Bio-Rad) using bovine serum albumin as the standard.

RESULTS

Overexpression and Large-Scale Purification of Recombinant hNTH1 K212R. In our previous report the full-length hNTH1 K212R cDNA was expressed using a pGEX-2T vector (*14*). The thrombin digestion of GST−K212R fusion protein resulted in a full-length and a truncated polypeptide lacking 22 residues in addition to the GST protein. The presence of the internal thrombin cleavage site in the hNTH1 protein resulted in significant loss of full-length K212R protein. Therefore, in this study we reconstructed the expression plasmid by inserting the full-length hNTH1 K212R cDNA into a pGEX-6P1 vector. The mutant protein was overexpressed as a fusion protein with GST at the N-terminus. On digestion with PreScission protease, the GST−K212R fusion protein showed a single hNTH1 K212R band on SDS−PAGE in addition to the expected band for GST protein (Figure 1). N-Terminal sequencing of the K212R band showed that the protein was hNTH1 with five additional N-terminal residues (Gly-Pro-Leu-Gly-Ser) derived from the vector. The yield of the final hNTH1 K212R protein was about 4−5 mg/1 L cells.

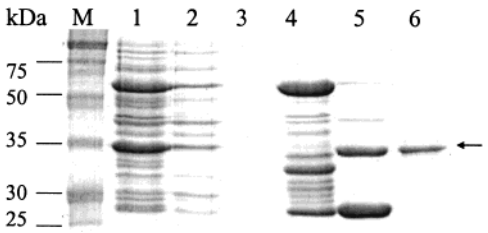

FIGURE 1: Expression and purification of the hNTH1 K212R mutant. The K212R mutant was analyzed by SDS−12% PAGE and Coomassie Blue staining. Lanes: M, protein markers; 1, total sonicated extract of *E. coli* harboring hNTH1 K212R expression vector induced with 0.1 mM IPTG for 4 h; 2, flow-through fraction from the glutathione−Sepharose column; 3, wash fraction from the glutathione−Sepharose column; 4, purified K212R protein after elution from glutathione−Sepharose with 15 mM glutathione; 5, the protein in lane 4 digested by PreScission protease; 6, K212R mutant completely separated by SP-Sepharose chromatography. The arrow indicates the predicted position of the mutant.

Cross-Linking of the Reaction Intermediate by $NaCNBH_3$ and Purification of the Covalent Complex. Previous study showed that although the hNTH1 K212R had much lower (~100-fold) DNA glycosylase/AP-lyase activity than the WT protein, the enzyme−DNA imino intermediate could be trapped by $NaCNBH_3$ (*14*). To identify the nucleophilic residue that forms the covalent linkage to the substrate DNA, the trapping reaction was carried out in large scale using the hNTH1 K212R protein and the radiolabeled 13-mer duplex oligonucleotide containing an AP site in the presence of $NaCNBH_3$. The cross-linked complex (~37 kDa) was separated from the free protein and oligonucleotide with an anion-exchange Q-Sepharose column (Figure 2A). The free protein did not bind to the column, whereas the complex was eluted in 0.15−0.3 M NaCl, leaving the free oligonucleotide to be eluted at higher salt concentration. The K212R−DNA complex was hydrolyzed exhaustively with endoproteinase Glu-C, and the hydrolysate was separated on a C18 reversed-phase HPLC column. The peptide fragments bearing the radiolabeled oligonucleotide were eluted at about 5% (fraction 5, 530 dpm), 6% (fraction 11, 3460 dpm), and 20% (fraction 25, 208 dpm) acetonitrile from the HPLC column (Figure 2B).

The results of microsequence analysis of the radioactive fractions are shown in Table 1. We did not observe loss of peak for any of the amino acid residues during sequencing analysis for any of the peptides. Therefore, we could not identify directly the residue(s) forming a covalent bond with the DNA. However, we consistently observed that only the N-terminal region of the K212R protein appeared in all of the radioactive peptides tested, and the sequencing reactions stopped invariably at proline 73 in three independent experiments (Table 1). The covalently bound DNA in that vicinity might create steric hindrance to Edman degradation during the sequencing reaction. Therefore, to identify the C-terminal end residue, the other aliquot of fraction 11 was subjected to mass spectrometry. The average mass of fraction 11 was 10420.00 ± 0.60. This value corresponds to the sum of the calculated masses of a duplex 13-mer oligonucleotide and 19 amino acid residues (^{62}GSDSEKGEGAEPLKVPVW^{80}E). Thus, we presume that Lys-75 in the nonsequenceable region (^{73}PL^{75}KVPVW^{80}E) is an "opportunistic" nucleophile that forms a covalent linkage to substrate DNA via a Schiff base intermediate.

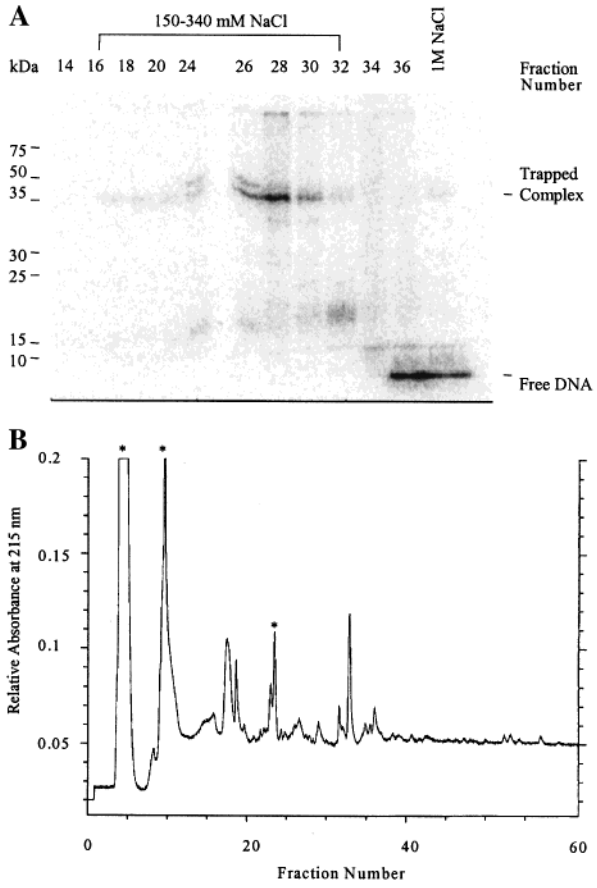

FIGURE 2: (A) Purification of trapped K212R 13 ds AP oligonucleotide on the Hitrap 5.0 mL Q-Sepharose column. The K212R mutant (about 90 nM) was allowed to react with [^{32}P]AP 13 ds oligonucleotide (60 nM) in the presence of 50 mM $NaCNBH_3$ at 37 °C for 2 h, and the trapped complex was separated as described in Materials and Methods. The fractions were analyzed by 12% SDS−PAGE. The gel was autoradiographed for visualization of the complex. (B) Separation of radioactive peptides by HPLC. The hNTH1 K212R (^{32}P-labeled) oligonucleotide covalent complex was digested with endoproteinase Glu-C and separated by HPLC as described under Materials and Methods. Peaks marked by asterisks contained the radioactivity, which was dried and used for N-terminal sequencing and mass spectrometric analysis.

Table 1: Amino Acid Sequence Analysis of the Peptide−Oligonucleotide Complexes[a] Derived from the hNTH1 K212R−DNA Covalent Complex

HPLC	amino acid sequence	C terminus
fractions 5 and 6	no signal	
fraction 11	^{62}GSDSEKGEGAE	~80[b]
fraction 25	^{62}GSDSEKGEGAE ^{67}KGEGAE ^{6}ARMLTR	

[a] Radiolabeled peptides derived from the hNTH1 K212R−DNA complex after digestion with Endo Glu-C and isolation by HPLC (Figure 3). [b] The C terminus was defined by mass spectrometry.

Site-Directed and Deletion Mutagenesis of K212R-hNTH1 at the N-Terminal Region. To test whether Lys-75 acts as an opportunistic nucleophile in the AP-lyase reaction, we directly substituted Lys-75 of K212R with Gln. The double site-specific mutant K212R-K75Q-hNTH1 was overexpressed as a GST fusion protein and purified similarly as the K212R protein (Figure 3). The double mutant retained the [4Fe-4S] cluster which is evident from its yellowish brown color. However, this double mutant did not lose

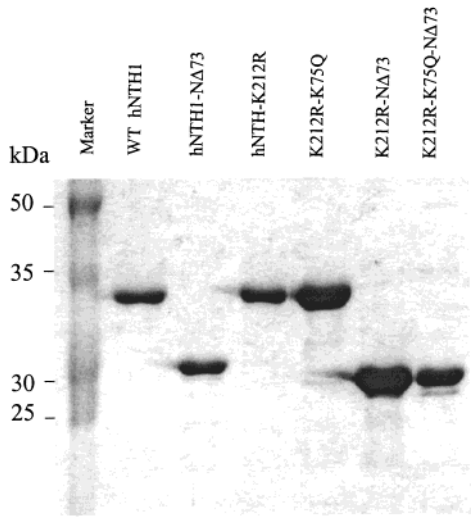

FIGURE 3: Purification of hNTH1 proteins analyzed by SDS−12% PAGE and Coomassie Blue staining.

glycosylase/lyase activity and in fact had similar glycosylase/lyase activity (Figure 3) and trapping efficiency (data not shown) as the single mutant, K212R protein.

There are five lysines (^{42}K, ^{48}K, ^{52}K, ^{64}K, and ^{75}K) present within the first 80 residues of the hNTH1 protein. Therefore, we cannot rule out the possibility that, in the absence of Lys-75, the other lysine(s) in the double mutant may also act as opportunistic nucleophile(s) and carry out the lyase activity. To test this possibility, we created three N-terminally truncated polypeptides, namely, hNTH1 NΔ72, hNTH1 K212R-NΔ72, and hNTH1 K212R-K75Q-NΔ72. All proteins including WT and mutants were purified to near homogeneity (Figure 3) and analyzed for absorbance at 410 nm. The results showing that all of the mutants have absorbance similar to that of the WT protein at similar protein (molar) concentrations indicate the presence of the intact [4Fe-4S] cluster.

Functional Analysis of Mutant hNTH1 Proteins. The WT and the mutant hNTH1 proteins were tested for both glycosylase and glycosylase/AP-lyase activities. To dissect the glycosylase activity from the concerted glycosylase/AP-lyase activities of different mutant proteins, we heated the reaction mixture in the presence of piperidine. Piperidine cleaved the leftover AP sites, if generated at DHU sites by the enzymes. Following a full time course of enzyme activity of WT and NΔ72-truncated hNTH1s under nonturnover conditions ([E] ≫ [S]; E:S = 16:1), we found that the rates of glycosylase and AP-lyase activities for both of the enzymes differed at earlier time points and merged at 10−12 min after initiation of the reactions (Figure 4A), indicating dissociation of a fraction of the Schiff base intermediate of the enzyme−substrate covalent complex during the early time points before its conversion to the single-strand cleaved product, induced by enzyme's AP-lyase activity. Other laboratories also made similar observations for NTH and OGG proteins (*23*−*25*).

The hNTH1 K212R or its subsequent mutants, hNTH1 K212R-NΔ72, hNTH1 K212R-K75Q, and hNTH1 K212R-K75Q-NΔ72, showed very low and nonreproducible activities at 240 nM concentration under nonturnover conditions. Therefore, we used a higher protein concentration (1 μM) and followed the full time course for both of the activities.

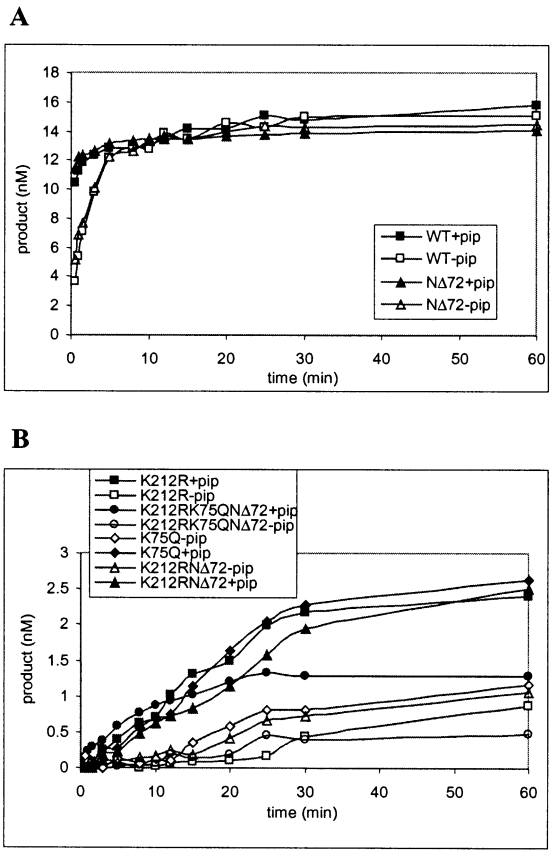

FIGURE 4: Comparison of functionality of WT and mutant proteins. (A) Fifteen nanomolar DHU-56 duplex oligonucleotide was incubated with 240 nM WT hNTH1 and hNTH1 NΔ72 proteins for the indicated times at 37 °C with or without subsequent treatment of 200 mM piperidine (±pip). (B) Fifteen nanomolar DHU-56 duplex oligonucleotide was incubated with 1 μM hNTH1 K212R, K212R-K75Q, K212R-NΔ72, or K212R-K75Q-NΔ72 proteins for the indicated times at 37 °C with or without subsequent treatment of 200 mM piperidine (±pip). Products were separated by denaturing gel electrophoresis and analyzed by a phosphorimager as described in Materials and Methods.

The results demonstrated that the rate of base removal by the mutants, K212R, K212R-NΔ72, K212R-K75Q, and K212R-K75Q-NΔ72, was always higher than that of AP-site cleavage (Figure 4B). At earlier time points (up to 10 min) the K212R, the K212R-K75Q, and the triple mutant showed no AP-lyase activity with detectable glycosylase activity, indicating complete uncoupling of these two activities. Although the differences in these two excision rates are clearly diminishing with time, the rates of these two activities for all four mutants, as opposed to wild-type enzymes, did not merge even at 60 min. Nonetheless, the triple mutant initially followed similar kinetics in both base removal and AP-site cleavage, but after 25−30 min neither of these activities of this mutant increased with time, suggesting higher susceptibility to inactivation of the mutant protein even in the presence of substrate DNA.

DISCUSSION

The hNTH1 is a DNA glycosylase/AP lyase and nicks DNA at the damage site as well as directly at the AP site (*14*). The chemistry of the strand cleavage event associated with the DNA glycosylase/AP lyases has been worked out in detail. In general, this group of glycosylases cleaves phosphodiester bonds via β-elimination by abstracting the *pro-S* hydrogen from the C-2′ position on the deoxyribose (*26*). We previously reported that hNTH1 like *E. coli* Nth performs this reaction via β-elimination (*14*). The C-2′ proton of a ring-opened abasic aldehyde will have a pK_a of approximately 18, and the abstraction of this proton by an active site base may not be favored (*21*). This can be accomplished by formation of an enzyme−DNA imino intermediate (Schiff base) involving the aldehyde of the ring-opened abasic site and an amine group from the enzyme. The amino group involved in this reaction scheme has been identified for a number of AP lyases by direct cross-linking to an AP site in DNA by reduction with borohydride and amino acid sequencing (*27*, *28*). Although no direct evidence was shown for the active site nucleophile (Lys-120 predicted from crystal structure and mutagenesis) of *E. coli* Nth, we showed by reductive cross-linking and direct sequencing that Lys-212 in hNTH1 formed a Schiff base with an abasic site in an oligonucleotide DNA (*14*). Furthermore, the site-specific substitution of Lys-212 of hNTH1 with Gln caused complete inactivation of the enzyme but did not affect the substrate DNA binding (*14*) as was also shown with an analogous mutant of *E. coli* Nth (*18*). However, the K212R mutant of hNTH1 was active although with a much lower k_{cat} and 85-fold less catalytic specificity than the WT protein (*14*). A similar situation was observed with the K241R mutant of yeast OGG1 (*29*). This prompted us to further investigate the properties of this K212R-hNTH1 mutant protein.

Since the K212R as opposed to the K212Q is active, the Arg directly or indirectly appears to be involved in catalysis. The nonionized form of the guanidino group would only be present at very small concentrations at physiological pH, however, is a strong nucleophile, and may attack the C1′ of the sugar to facilitate the cleavage of the glycosylic bond and displace the modified base. The Schiff base formed between the guanidino group of Arg and deoxyribose should rapidly hydrolyze before the β-elimination event. Thus the Arg-mediated Schiff base imino intermediate may not be trapped by reduction with borohydride. In fact, our borohydride cross-linking coupled with sequencing of the imino intermediate complex of K212R showed only the presence of Lys-rich N-terminal region peptides, suggesting the involvement of opportunistic lysine(s) of the N-terminal region and exclusion of Arg-212 in the β-elimination event. Although we cannot rule out the possibility that, instead of the guanidino group of Arg directly catalyzing glycosylic bond cleavage, the highly basic Arg is holding a water molecule in a compromised catalytic pocket of the K212R mutant and that water molecule being activated by the Asp-231 may attack the C1′ of the glycosylic bond for cleavage. In either case the glycosylase reaction will be followed by a secondary β-elimination event carried out by a random Lys residue in the K212R protein. In fact, the triple mutant lacking the N-terminal lysines retains similar AP-lyase activity as the K212R (Figure 4B), probably using some random lysine(s) from the core region.

These results with the K212R-hNTH1 mutant are very similar to the properties of WT MutY, which utilizes a hydrolytic attack for its glycosylase activity, and forms a covalent Schiff base with substrate DNA (*30*, *31*). There were controversies for its low AP-lyase activity, but it was shown

recently that opportunistic lysines 142 (major) and 132 (minor) are participating in Schiff base formation and are responsible for reductive cross-linking between MutY and substrate DNA (*31*).

The K212R and its subsequent mutants showed glycosylase activity but could not demonstrate detectable AP-lyase activity during early time points (Figure 4B), indicating that the substitution of Lys-212 with Arg completely uncoupled glycosylase activity from its associated AP-lyase activity. The significant delay in initiating the AP-lyase activity in these mutants may be due to the secondary β-elimination reaction by random lysine residues including release and rebinding of the enzyme. This observation again mimics the properties of MutY. The substitution of Lys-142, the opportunistic nucleophile, with Ala did not inactivate the glycosylase activity (*32*). Therefore, the glycosylase and AP-lyase activities of WT MutY were also suggested to be uncoupled (*30*). Thus the underlying mechanism of uncoupled reaction of K212R and MutY is probably similar in utilizing two different catalytic centers: an activated water molecule or guanidino group of Arg in K212R for the primary glycosylase reaction and a random Lys for the secondary β-elimination event. On the other hand, WT hNTH1 may follow a true coupled reaction, which based on mechanistic considerations should involve a single catalytic center for both the glycosylase and the β-elimination reaction; an activated Lys attacks the deoxyribose at C1′ of the lesion, causing release of the modified base and forms a Schiff base, which has the potential to be involved in subsequent β-elimination for producing a strand break. The Schiff base can also be hydrolyzed prior to the β-elimination event by hydrolysis (*19*).

It is important to note that, unlike hNTH1, *E. coli* Nth does not have the extra N-terminal region consisting of 95 residues. It will be interesting to know whether the K120R mutant of *E. coli* Nth has AP-lyase activity.

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