**In Vitro and in Vivo Dimerization of Human Endonuclease III Stimulates Its Activity**

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Human endonuclease III (hNTH1), a DNA glycosylase with associated abasic lyase activity, repairs various mutagenic and toxic-oxidized DNA lesions, including thymine glycol. We demonstrate for the first time that the full-length hNTH1 positively cooperates in product formation as a function of enzyme concentration. The protein concentrations that caused cooperativity in turnover also exhibited dimerization, independent of DNA binding. Earlier we had found that the hNTH1 consists of two domains: a well conserved catalytic domain, and an inhibitory N-terminal tail. The N-terminal truncated proteins neither undergo dimerization, nor do they show cooperativity in turnover, indicating that the homodimerization of hNTH1 is specific and requires the N-terminal tail. Further kinetic analysis at transition state reveals that this homodimerization stimulates an 11-fold increase in the rate of release of the final product, an AP-site with a 3'-nick, and that it does not affect other intermediate reaction rates, including those of DNA N-glycosylase or AP lyase activities that are modulated by previously reported interacting proteins, YB-1, APE1, and XPG. Thus, the site of modulating action of the dimer on the hNTH1 reaction steps is unique. Moreover, the high intranuclear (2.3 μM) and cytosolic (0.65 μM) concentrations of hNTH1 determined here support the possibility of *in vivo* dimerization; indeed, *in vivo* protein cross-linking showed the presence of the dimer in the nucleus of HeLa cells. Therefore, it is likely that the dimerization of hNTH1 involving the N-terminal tail masks the inhibitory effect of this tail and plays a critical role in its catalytic turnover in the cell.

Reactive oxygen species are generated endogenously in cells as byproducts of oxidative phosphorylation, and enzymatically during inflammatory responses and detoxification reactions. Reactive oxygen species are also generated exogenously because of metal toxicity, ionizing radiation, or in the influence of other environmental agents; reactive oxygen species are by far the most important genotoxic agents that cause various mutagenic, carcinogenic, and cytotoxic lesions in DNA (1). Oxidative DNA damage includes single- and double-strand breaks and a myriad of base lesions. The oxidized base lesions, known to date, are removed from DNA by the base excision repair (BER1) pathway. This process is initiated by bifunctional DNA glycosylases that not only catalyze removal of the base lesion but also cause strand cleavage at the resulting apurinic/apyrimidinic (AP) site via β-elimination by their associated AP lyase activity (2–4). Among such oxidatively damaged bases, a series of structurally diverse toxic or mutagenic oxidized pyrimidines are repaired by endonuclease III (NTH), a glycosylase/AP lyase present in all species from bacteria through man. Subsequent repair steps include removal of the resulting 3′ α,β-unsaturated aldehyde by the phosphodiesterase activity of AP-endonuclease (APE), filling of the resulting DNA gap by a DNA polymerase, and, finally, sealing of the repaired strand by DNA ligase (4, 5).

The NTH family of repair glycosylases/lyases constitutes a conserved class of enzymes that seem to be present throughout phylogeny (4). The *Escherichia coli* endo III recognizes a wide range of oxidized pyrimidine derivatives, including ring-saturated and ring-fragmented derivatives such as thymine glycol (Tg), 5-hydroxycytosine, dihydrouracil (DHU), uracil, and at least six other oxidized pyrimidines (2, 4, 6–8). 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 (9). Structural analysis of *E. coli* and archaeal Nths, by x-ray crystallography and heteronuclear NMR spectroscopy, respectively, reveals that those repair enzymes consist of two α-helical domains that contain five DNA binding motifs: helix-hairpin-helix (HhH), [4Fe-4S]2−-unsaturated

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1 The abbreviations used are: BER, base excision repair; AP, apurinic/apyrimidinic; DHU, dihydrouracil; hNTH1, human endonuclease III; Nth, *E. coli* endonuclease III; Tg, thymine glycol; APE, apurinic/apyrimidinic endonuclease; WT, wild type; DSS, disuccinimidyl suberate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; YB-1, Y-box binding protein 1.

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overall catalytic turnover of hNTH1 by reducing the product release rate 5–7-fold (16). Earlier we had found that the size of hNTH1 in human whole cell extract is similar to that of purified recombinant full-length protein on Western blot analysis (13). This finding indicated that, most likely, hNTH1 in human cells carries the N-terminal tail. Therefore, in an in vitro situation, the cell probably minimizes this apparent inhibitory effect of the N-terminal tail during catalysis. One obvious way could be by masking the N-terminal tail through protein-protein interactions.

Here we demonstrate that hNTH1 forms a dimer in vivo and in vitro involving the N-terminal tail. Moreover, this homodimerization indeed masks the inhibitory effect of this tail, and enhances the rate of product release, which is the limiting step in the multistep reaction process of hNTH1.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of WT and the Truncated hNTH1s—**The full-length and truncated (N555, N572, and N580) hNTH1 proteins were expressed as glutathione S-transferase fusion and purified to near homogeneity as described earlier (16). The yield of hNTH1 proteins was about 10–12 mg/liter of E. coli culture; these were stored in aliquots at –80°C. Oligonucleotide Substrates—A DHU-containing 56-mer oligonucleotide (DHU-56) with the sequence 5′-ATTATGCTGAGTGATATC-CCTCTGGCCTTCGAACCCC (where X represents DHU) was purchased from Operon Technologies (Alameda, CA). The complementary oligonucleotide containing A opposite DHU was synthesized by the Recombinant DNA Core Facility at the University of Texas Medical Branch, Galveston, TX. The oligonucleotides were purified on a sequencing gel. The DHU-56 oligonucleotide was labeled at the 5′-end using T4 polynucleotide kinase and [γ-32P]ATP, and was annealed to complementary oligonucleotide (DHU-56: complementary = 1:1.7) to prepare 32P-end-labeled duplex DHU-56 oligonucleotide as described previously (17).

**Enzyme Cleavage Assay—**The 5′-end-labeled oligonucleotide substrate (DHU-56) and cold DHU-56 (2 μM) were incubated with the hNTH1 proteins (10–400 nM) at 37°C for 10 min in a reaction mixture (10 μl) containing 50 mM HEPES, pH 7.9, 75 mM NaCl, and 1 mM dithiothreitol. After the reaction was halted with 25 μl of stop solution (90% formamide, 0.025% bromphenol blue, 0.025% xylene cyanol, and 4% glycerol), the mixtures were heated at 95°C for 5 min, and then 5 μl of each 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 (Amersham Biosciences) and subsequently the data were processed as described earlier (13, 16).

**Single Turnover Assay—**The assay was performed as described previously with WT and truncated hNTH1 proteins (6.7 or 67 pmol) and 5′-32P-end-labeled DHU-56 substrate oligonucleotide (about 300 pmol or 3 pmol duplex; Ref. 16). Briefly, the assay was performed at 37°C in a buffer containing 50 mM HEPES, pH 7.9, 75 mM NaCl, 0.1 mg/ml bovine serum albumin, 0.5 mM EDTA, and 1 mM dithiothreitol. The proteins were diluted to working conditions in assay buffer and equilibrated at 37°C. Twenty-nine μl of 5′-32P-end-labeled DHU-56 oligonucleotide were mixed with 29 μl of 10× assay buffer and 203 μl of H2O. After equilibration at 37°C for 5 min, a 9-μl aliquot was removed as control before adding 28 μl of hNTH1 or truncated proteins to make a final volume of 280 μl. To measure strand cleavage, two 10-μl aliquots were removed at different time periods (0–60 min). All aliquots were immediately treated with 25 μl of loading dye (95% deionized formamide, 10 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol) and heated at 95°C for 3 min. To measure glycosylase activity, one aliquot was treated with 200 mM piperdine prior to heating at 95°C for 5 min.

Radioactivity in the incised oligonucleotide was quantified by exposing the gel to a PhosphorImager (Amersham Biosciences).

**“Burst” Analysis—**This assay was performed as described previously with a final concentration of the duplex DHU-56 substrate oligonucleotide, 35 nm or 2 μM at a ratio of unlabeled to labeled of 5:1, and WT and truncated proteins (6 or 200 nM) (Ref. 16). Briefly, 29 μl of 10× assay buffer, unlabeled DHU-56 oligonucleotide, and 29 μl of 5′-32P-end-labeled DHU-56 oligonucleotide were mixed to a total volume of 261 μl. An aliquot of 9 μl was removed as control prior to addition of 28 μl of the different proteins. Finally, 10-μl aliquots were removed at different time periods (0–60 min). All aliquots were immediately treated with 25 μl of loading dye and processed for gel and PhosphorImager analysis as described above. Data obtained from the single and multiple turnover kinetic assays were weighted using GraphPad Prism 3.0 software (GraphPad Software, Inc., San Diego, CA).

**In Vivo Protein-Protein Cross-linking—**In vitro cross-linking of purified hNTH1 WT and truncated proteins with a homobifunctional non-cleavable cross-linker, disuccinimidyl suberate (DSS, Pierce) dissolved in Me2SO, was performed with various concentrations of proteins (50 nM to 1 μM) and 0.5 mM DSS in Dulbecco’s phosphate-buffered saline (PBS; 1.5 mM KH2PO4, 8 mM Na2HPO4, 2.7 mM KCl, and 138 mM NaCl; Invitrogen) at room temperature for 30 min. Instead of DSS, only Me2SO was added for control experiments. To assess the effect of DNA on dimerization, cross-linking was performed similarly using 400 nM WT protein and 500 nM DHU containing or control DNA substrates. Cross-linked products were detected by Western blotting using an affinity purified anti-hNTH1 polyclonal antibody and the enhanced chemiluminescence (ECL) technique as described earlier (13, 16).

**For Western Blotting—**The expression vector, pGEX-2TK (Amersham Biosciences), carrying full-length hNTH1 cDNA, was used to express the derivative of hNTH1 that carries a N-terminal 4-amino acid sequence (RRASV). This specific sequence allows the protein to be radiolabeled with [γ-32P]ATP. The protein was expressed and affinity purified from E. coli as described earlier (16). The RRASV-tagged hNTH1 was then radiophosphorylated in vitro (18) to specific activities of 1–6 × 106 cpm/μg of protein. The WT and N580 hNTH1 proteins in various concentrations, as indicated, were either spotted directly on nitrocellulose filter, or subjected to conventional SDS-PAGE prior to their transfer to a nitrocellulose filter. The filters were then processed for binding with [32P]-labeled WT hNTH1 (2.5–2.5 × 106 cpm/ml) in a hybridization buffer without the probe for 7, 5, and 2 min, the blots were analyzed by exposing them to x-ray film as described by Hale and de Boer (19).

**Quantitative Determination of Intracellular hNTH1 Concentration—**hNTH1 concentration was measured both in the nuclei and cytosol of HeLa cells. The cells were grown to ~90% confluence in six 100-mm plates, washed with Dulbecco’s PBS, and trypsinized. Cells (~1.5 × 106) from 2 plates were taken up in 10 ml of PBS and counted for total cell number and viability by the trypan blue exclusion method. More than 98% of the cells were viable. Then the nuclear and cytosolic extracts were prepared from each of three replicates of cellular pools using the NE-PER extraction kit (Pierce). Various amounts of proteins in both cytosolic and nuclear extracts as well as purified hNTH1 protein, as indicated, were analyzed by SDS-PAGE, followed by Western blotting using anti-hNTH1 polyclonal antibody and the ECL technique described earlier (16). Intensities of the purified hNTH1 protein bands were quantitated and the data were analyzed using GraphPad Prism™ version 4.0 and fitted to a sigmoid regression curve-fit model to generate the standard curve (r2 = 0.98). The unknown concentrations of hNTH1 in nuclear and cytosolic extracts from three replicates of cellular pools were then interpolated separately from the standard curve using the automated interpolation mode of Prism™. Finally, the molar hNTH1 concentration was estimated per nucleus and cytosolic space per cell, on the basis of published average nuclear and total cell volume of a single HeLa cell (20, 21).

**In Vivo Protein-Protein Cross-linking by Formaldehyde—**Proteins were cross-linked in vivo by incubating HeLa cells in various concentrations (as indicated) of formaldehyde as described by Bakkenist and Krokan (22) except in Dulbecco’s modified Eagle’s medium (without fetal bovine serum) instead of PBS for 10 min at room temperature. Formaldehyde was washed out using PBS that contained 100 μM glycine, before preparing the nuclear and cytosolic extracts with the aid of 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5 M NaCl, and 1 mM dithiothreitol. The extracts were analyzed by gel electrophoresis and Western blotting using anti-hNTH1 polyclonal antibody and the ECL technique described previously (16).

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

**RESULTS**

**hNTH1 Exhibits Non-Michaelis-Menten Kinetics—**In our previous study (16), we showed unequivocally that the rate of release of hNTH1 from its own reaction product is extremely slow (0.0043 min−1 with a t1/2 of 2.7 h) indicating that the
product release is rate-limiting and significantly responsible for the slow turnover of this enzyme. Nonetheless, this extremely low rate also revealed that standard steady state kinetics (Michaelis-Menten) of hNTH1 would require extremely long reaction times and hence may not be meaningful. In this study, we further investigated this issue and tested the effect of increasing concentrations of hNTH1 on the cleavage rate of DHU-containing oligonucleotide substrate. Fig. 1A shows that the product formation by the WT protein is not linear with increasing concentrations of protein, suggesting positive cooperativity (non-Michaelis-Menten kinetics) in the processing of DHU by hNTH1. A recent report documented the same phenomenon for hNTH1 with Tg as a substrate (23). Likely, the homodimerization of hNTH1 facilitates enzyme function and shows apparent, positive cooperativity.

**Fig. 1. Effect of increasing concentrations of hNTH1 on its excision activity for DHU-containing duplex DNA substrate.** Experimental details are under “Experimental Procedures.”

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**Fig. 2. Stabilization of the hNTH1 dimer by in vitro protein-protein cross-linking with DSS and effect of DNA on in vitro dimerization of hNTH1.** A, cross-linking of various concentrations of WT hNTH1 and detection of the WT dimer and monomer by SDS-PAGE and immunoblotting are described in detail under “Experimental Procedures.” B, the dimeric concentration of hNTH1 was determined based on band intensities for monomer and dimer and plotted against hNTH1 protein concentration. C, effect of DNA on in vitro dimerization of the hNTH1. Cross-linking was performed as described in A with 400 nM WT NTH1 and 500 nM control or DHU-containing duplex DNA.

hNTH1 Exhibits Homodimerization in Solution in Vitro—To test this possibility, we attempted solution cross-linking, a very sensitive and widely used method (24, 25) for in vitro analysis of protein-protein interactions in solution, to trap the hNTH1 homodimer. We found that a homobifunctional, non-cleavable chemical cross-linker, DSS, cross-linked hNTH1 as a function of protein concentration (Fig. 2, A and B). The cross-linked species were identified by Western blot analysis with antibodies specific to the hNTH1 protein. The apparent size (~68 kDa) of this complex in SDS-PAGE suggests that this complex corresponds to a homodimer of the hNTH1 protein (32–34 kDa when monomer). Notably, protein concentrations (>100 nM) that showed positive cooperativity in turnover (Fig. 1) also exhibited formation of homodimer in a similar fashion (Fig. 2, A and B). This dimerization of hNTH1 is specific because ear-
liyer we found that other DNA glycosylases, namely, N-methylpurine DNA-glycosylase, when treated with the cross-linking agent never showed any dimer, and remained as monomers (26). Furthermore, the extent of cross-linking was not affected by either control or substrate DNA (Fig. 2C). Therefore, hNTH1 forms a dimer in solution and, unlike adenine DNA glycolysase, MutY from *E. coli* does not require substrate DNA. A band of smaller size than the monomer was generated after addition of DSS. The smaller size species could have been generated because of degradation or faster migration on SDS-PAGE of a fraction of hNTH1 molecules as a result of intramolecular cross-linking and compaction of protein structure. Notably, the segregation of the monomer proteins into various separable forms on SDS-PAGE upon treatment with chemical cross-linker is not unusual (27).

The N-terminal Tail of hNTH1 Serves an Important Role in Homodimerization and Positive Cooperativity—Because we have shown earlier that the N-terminal tail inhibits the turnover of hNTH1 significantly (16), we have asked in this study whether the N-terminal tail has any role in homodimerization and positive cooperativity. To investigate the role played by the N-terminal tail of hNTH1 protein in homodimerization, we tested the ability of several hNTH1 derivatives, bearing nested deletions of N-terminal sequences, to form dimers in the *in vitro* solution cross-linking assay with DSS. Fig. 3 shows that the WT protein was cross-linked as before to form a dimer. The truncation of 55, 72, and 80 residues from the N terminus reduced the complex formation 1.7-, 3.2-, and 3.6-fold, respectively. Thus, the extent of cross-linked dimer formation decreased with successive truncation of N-terminal tail residues, indicating that the homodimerization of hNTH1 is specific and involves the N-terminal tail.

**Table 1.**

| Complex (%) | 0 | 29 | 17 | 0 | 9 | 0 | 8 |
|-------------|---|----|----|---|---|---|---|
| hNTH1 WT    | - | +  | -  | - | - | + | - |
| hNTH1 NΔ55  | - | +  | -  | - | - | + | - |
| hNTH1 NΔ72  | - | +  | -  | - | - | + | - |
| hNTH1 NΔ80  | - | +  | -  | - | - | + | - |

DSS reacts with primary amines of a protein and obviously requires lysines. Indeed, the first 80 residues of the tail contain five lysines (Lys<sup>42</sup>, Lys<sup>48</sup>, Lys<sup>52</sup>, Lys<sup>64</sup>, and Lys<sup>75</sup>). One may argue that the observed reduction in cross-linking with successive truncation of the N-terminal tail residues actually accounts for the loss of lysines and, thus, the DSS-cross-linked complex of the hNTH1 dimer is an artifact. Therefore, we tested this homointeraction between hNTH1s by another sensitive technique, namely, Far Western blotting (18, 19, 24). We prepared derivatives of hNTH1 that carry an N-terminal 4-amino acid sequence (RRASV), allowing the protein to be radiolabeled with [γ-<sup>32</sup>P]ATP.

These additional residues did not affect the enzyme activity (data not shown). We then used this [γ-<sup>32</sup>P]-labeled hNTH1 to probe WT and NΔ80 hNTH1s on the same blot. Although we electrophoresed equimolar amounts (9.5–152 pmol of each protein and 72 pmol for BSA) of these two proteins (as evident from Ponceau S-stained proteins on nitrocellulose membrane; Fig. 4A), the [γ-<sup>32</sup>P]-labeled WT probe bound WT protein with at least 3-fold greater affinity than the NΔ80 hNTH1 in a concentration-dependent manner (Fig. 4B and C). Because this experiment requires renaturation of SDS-denatured proteins on nitrocellulose membrane prior to binding with [γ-<sup>32</sup>P]-labeled WT hNTH1 probe, one concern is that the difference in binding affinity of the WT probe for WT and NΔ80 could be because of differences in the extent of renaturation of those two membrane-bound proteins. To avoid the renaturation process, we directly spotted WT and NΔ80 at a range of 9.5–152 pmol along with BSA (72 pmol) as control onto a nitrocellulose membrane with a slot-blot apparatus and probed with [γ-<sup>32</sup>P]-labeled WT hNTH1. Here too the [γ-<sup>32</sup>P]-labeled WT probe bound WT protein with at least 3–4-fold greater affinity than the NΔ80 hNTH1 in a concentration-dependent manner (Fig. 5A, A and B). On the other hand, BSA did not show any significant binding in Fig. 4B, except for some binding, similar to NΔ80, albeit with at least 4-fold reduced affinity compared with the WT protein in Fig. 5A. The binding with BSA and NΔ80 may be regarded as nonspecific background binding. Thus, Far Western blotting confirmed that the homointeraction of hNTH1 is specific and involves the N-terminal tail.

Then we tested the activity of these N-terminal-truncated proteins along with the WT as a function of protein concentration and found that up to 100 nM of the truncated proteins (NΔ72 and NΔ80) show higher activity than the WT protein.

![Fig. 3. Role of the N-terminal tail in stabilization of hNTH1 dimer by in vitro protein-protein cross-linking with DSS. WT and truncated hNTH1s were incubated at higher protein concentrations (2 μg) with 0.5 mM DSS. Other experimental conditions are the same as described in the legend to Fig. 2.](image-url)
and NΔ55 in a linear fashion, but at a higher protein concentration (>100 nM) the curve became sigmoid for WT, as we observed in Fig. 1 and also for NΔ55 (data not shown). This nonlinearity diminished gradually with successive truncation of N-terminal tail residues beyond 55, and, finally, the rate of product formation became saturated at higher protein concentrations (300–400 nM) for NΔ80 and NΔ72 as opposed to WT and NΔ55 (data not shown). Thus, the N-terminal tail plays a role in the homodimerization of hNTH1 in a concentration-dependent manner, and this protein-protein interaction may be a plausible mechanism for the positive cooperativity displayed by the WT hNTH1. Moreover, our results show that homodimerization may be the mechanism by which the inhibitory effect of the N-terminal tail is overcome.

**Inhibition of Activity of hNTH1 at Low Concentration Depends on the N Terminus and Is Released by Homodimerization**—To elucidate how homodimerization of hNTH1 at high protein concentrations involves the N-terminal tail stimulates the activity of hNTH1, we tested the major rate constants at the intermediate steps of the reaction process for general DNA glycosylase/AP lyase-type enzymes as proposed by Gerlt (28) and Dodson et al. (29). We and others later applied this to hNTH1 and extended the investigation (16, 23). A previously proposed kinetic scheme to analyze the kinetic properties of hNTH1 is shown in Fig. 8. We have also used multiple and single turnover kinetics to determine the relevant rate constants (k₂, k₃, and k₅; 16), and found that, under multiple turnover conditions ([DHU-DNA]₅ >> [hNTH1]₁₂), the reaction of hNTH1 exhibits biphasic kinetics, consistent with a rate-limiting product release rate (k₅; 16). The rate constant for the exponential phase is proportional to the rate constants of the steps involving chemistry (k₂ and k₃), whereas the amplitude of the burst phase is proportional to the concentration of active hNTH1. The linear steady state portion of the plots is dominated by the rate of product release (k₅).

**Single Turnover Kinetics**—Prompted by the observation that homodimerization of hNTH1 stimulates its activity, we conducted single turnover experiments with WT and N-terminal-
truncated hNTH1 proteins, NAΔ55 and NAΔ80, which have significantly reduced the dimerization potential (Figs. 3–5) measuring the rate constants of base release ($k_2$) and β-elimination ($k_3$). The reactions were performed at similar ratios (1:24) of substrate (DHU) to enzyme concentrations but at varying enzyme concentrations of 24 and 240 nM, as the dimerization of hNTH1 is concentration-dependent (Figs. 2, 4, and 5). The $k_2$ was measured by treating the assay mixture with piperidine to cleave any residual AP site, formed by DNA glycosylase activity but not processed by the AP lyase activity of hNTH1; $k_3$ was directly measured by assaying the reaction product, which was produced by concerted DNA glycosylase/AP lyase reactions of hNTH1. Under these conditions, the observed rate constants ($k_2$ and $k_3$) remained unchanged (Table I). Neither truncation nor protein concentrations that facilitate homodimerization could alter the DNA glycosylase or the AP lyase activities of these proteins (Table I). Notably, the $k_2$ and $k_3$ differed 20-fold for both WT and truncated hNTH1s and these differences were not affected by protein concentrations. This is consistent with the published results, and could be because of significant hydrolysis of the Schiff base intermediates (16). In this report, we present evidence that hNTH1 forms a dimer in vivo and in vitro involving its N-terminal tail in a concentration-dependent manner. We also show that increasing protein concentrations lessens the apparent inhibitory effect of this tail and enhances the rate of product release, which is initially a limiting step in the multistep reaction process of hNTH1. To our knowledge, this is the first evidence that hNTH1 forms a dimer as a regulatory mechanism for stimulating its own activity.

In our previous study (16) we had shown that the rate of release ($k_2$) of hNTH1 from its own reaction product is extremely slow ($0.0043 \text{ min}^{-1}$ with a $t_{1/2}$ of 2.7 h), indicating that the product release is rate-limiting and significantly responsible for the slow turnover of this enzyme. The slow turnover is a common feature of mammalian DNA glycosylases including N-methylpurine DNA-glycosylase, OGG1, and TDG (14, 32, 33) in an in vitro reaction. In addition, analyses of the reactions of hUDG (34), E. coli UDG (35), hOGG1 (14, 36), mMYH (37), E. coli MutY (38), and hNTH1 (16) reveal a common theme for this class of enzymes, namely, that the overall rate for the catalytic cycle is controlled by a distinct step, following base removal, as evidenced by characteristic burst kinetics for product formation (16). In all cases, including hNTH1, the rate-limiting step is dominated by the rate of the release of the enzyme from the DNA product.

The slow rate of release of these enzymes from their reaction products could be envisioned as an advantage for the cell in preventing formation of unprotected DNA strand breaks. Notably, the phosphodiesterase activity of hAPE is very weak and is rate-limiting in the NTH-mediated BER pathway (39). The 3’ α,β-unsaturated aldehyde at the single strand break created by hNTH1 needs to be repaired by hAPE to provide appropriate

## Table I

| Protein type | Protein | Substrate | $n_M$ | $k_2$, min$^{-1}$ | $k_3$, nM$^{-1}$ |
|--------------|---------|-----------|------|-----------------|----------------|
| Wild type    | 24      | 1         | 9.7  | 0.55 ± 0.04     |                |
|              | 240     | 10        | 11   | 0.50 ± 0.04     |                |
| NAΔ55        | 24      | 1         | 12.1 | 0.40 ± 0.06     |                |
|              | 240     | 10        | 12   | 0.60 ± 0.06     |                |
| NAΔ80        | 24      | 1         | 9.5  | 0.44 ± 0.07     |                |
|              | 240     | 10        | 10.5 | 0.60 ± 0.06     |                |

Homodimerization of hNTH1

In search of the mechanism of stimulation of activity of hNTH1 by homodimerization, we then attempted to measure the rate of product release ($k_2$). The extremely slow turnover rate of hNTH1 during the excision of DHU provided the opportunity to perform the burst analysis under reaction conditions of [SI] > [E], where the substrate and enzyme concentrations were 35 and 6 nM, respectively (30, 31). The reaction was also performed with increased concentrations of oligo and hNTH1 to assess the effect of homointeraction on $k_2$, without much alteration of substrate and enzyme concentration ratios. As observed earlier, at lower concentrations of hNTH1s (6 nM; 16) the $k_2$ (0.0043 ± 0.0005 min$^{-1}$) of WT hNTH1 is extremely low, apparently rate-limiting in the hNTH1-mediated multistep reaction process, and truncation of the N-terminal tail by 55–80 residues clearly increases the $k_2$ of hNTH1 at least 5–7-fold (Table II). Notably, the higher concentrations (200 nM) of hNTH1s caused a remarkable 11-fold increase in the rate of product release ($k_2$) for WT, but not for the N-terminal-truncated proteins, and it had a minimal effect on the rate or amplitude of the burst. Thus, these results demonstrate that the concentration-dependent homodimerization of hNTH1 involving the N-terminal tail seems to regulate its turnover by modulating product release ($k_2$) as the apparent rate-limiting step.

High Intracellular Concentration of hNTH1 Facilitates Its Dimerization in Vivo—Next we tested whether the high hNTH1 concentrations required in vitro to observe the dimer and its stimulating effect on its activity are biologically relevant. We directly determined the intracellular concentration of hNTH1 in both nuclei and cytosol of HeLa cells. Using immunofluorescence studies we previously showed that hNTH1 is present in both cytosol and nuclei of HeLa cells (13). Here we prepared both cytosolic and nuclear extracts from a known number of HeLa cells and quantified the amount of hNTH1 by Western blotting using various amounts of purified hNTH1 proteins as standard. The semiquantified hNTH1 concentration was then expressed per nucleus or cytosolic space based on the reported HeLa average nuclear (0.84 pl) or cytosolic (3.16 pl) volume, the latter was deduced by subtracting the nuclear volume from total cell volume (4 pl) of a single HeLa cell (20, 21). The results showed that the single HeLa nucleus contains significantly high hNTH1 concentration (2.3 ± 0.4 μM), whereas the cytosol contains merely a modest concentration (0.65 ± 0.4 μM), as the cytosol contains a large concentration of hNTH1. In our in vitro results showed that dimerization requires >100 nM in both nuclear and cytosolic concentrations; this suggests possible dimerization of hNTH1 in vivo.

**Discussion**

In this report, we present evidence that hNTH1 forms a dimer in vivo and in vitro involving its N-terminal tail in a concentration-dependent manner. We also show that increasing protein concentrations lessens the apparent inhibitory effect of this tail and enhances the rate of product release, which is initially a limiting step in the multistep reaction process of hNTH1. To our knowledge, this is the first evidence that hNTH1 forms a dimer as a regulatory mechanism for stimulating its own activity.

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In our previous study (16) we had shown that the rate of release ($k_2$) of hNTH1 from its own reaction product is extremely slow ($0.0043 \text{ min}^{-1}$ with a $t_{1/2}$ of 2.7 h), indicating that the product release is rate-limiting and significantly responsible for the slow turnover of this enzyme. The slow turnover is a common feature of mammalian DNA glycosylases including N-methylpurine DNA-glycosylase, OGG1, and TDG (14, 32, 33) in an in vitro reaction. In addition, analyses of the reactions of hUDG (34), E. coli UDG (35), hOGG1 (14, 36), mMYH (37), E. coli MutY (38), and hNTH1 (16) reveal a common theme for this class of enzymes, namely, that the overall rate for the catalytic cycle is controlled by a distinct step, following base removal, as evidenced by characteristic burst kinetics for product formation (16). In all cases, including hNTH1, the rate-limiting step is dominated by the rate of the release of the enzyme from the DNA product.

The slow rate of release of these enzymes from their reaction products could be envisioned as an advantage for the cell in preventing formation of unprotected DNA strand breaks. Notably, the phosphodiesterase activity of hAPE is very weak and is rate-limiting in the NTH-mediated BER pathway (39). The 3’ α,β-unsaturated aldehyde at the single strand break created by hNTH1 needs to be repaired by hAPE to provide appropriate concentration-dependent heterodimerization of hNTH1 and hAPE.
substrates to downstream BER enzymes to complete the repair process. Otherwise, imbalance in repair steps may lead to accumulation of strand breaks. Therefore, the minimizations of unprotected AP sites and/or strand breaks, generated by hNTH1 may aid in better regulation and thus benefit the cell. In fact, the low activity and product inhibition are not only general features of DNA glycosylases but are also true for other mammalian BER enzymes. hAPE was also shown to be structurally optimized to retain the DNA product containing a cleaved AP site; moreover, substitution of residues that penetrate the DNA helix stimulated the endonuclease activity of APE (40). In reality, each intermediate in BER is in itself a form of damage and evolution has designed a way to keep it from being exposed as such. Although individual enzymatic rates may be slow, in the cell the handoffs will presumably increase the rate of turnover (41). In fact a recent study showed that APE1, the enzyme next to hNTH1 in the BER pathway, stimulates the turnover of hNTH1 without interacting physically (42).

It is intriguing to observe that the apparent turnover of hNTH1 was stimulated at physiologically relevant higher protein concentrations. Fig. 1 shows that the product formation by wild type protein is not linear with increasing concentrations of protein, suggesting positive cooperativity (non-Michaelis-Menten kinetics) in the processing of DHU by hNTH1. A recent report confirmed this phenomenon for hNTH1 using Tg as a substrate (23). Positive cooperativity is a phenomenon displayed by various allosteric enzymes involved in different biochemical pathways. For example, glycogen phosphorylase is activated by various allosteric effectors through a dimerization event and shows positive cooperativity. Furthermore, the first 20 residues of this protein form a disordered region in the inactive form, whereas their phosphorylation achieves better order in the active form (30). In an analogous situation, hNTH1 at a protein concentration (>100 nM) showing positive cooperativity (Fig. 1), exhibits dimerization (Fig. 2). It is also intriguing that the first 100 residues comprising the N-terminal tail of hNTH1 may aid in better regulation and thus benefit the cell. In fact, the low activity and product inhibition are not only general features of DNA glycosylases but are also true for other mammalian BER enzymes. hAPE was also shown to be structurally optimized to retain the DNA product containing a cleaved AP site; moreover, substitution of residues that penetrate the DNA helix stimulated the endonuclease activity of APE (40). In reality, each intermediate in BER is in itself a form of damage and evolution has designed a way to keep it from being exposed as such. Although individual enzymatic rates may be slow, in the cell the handoffs will presumably increase the rate of turnover (41). In fact a recent study showed that APE1, the enzyme next to hNTH1 in the BER pathway, stimulates the turnover of hNTH1 without interacting physically (42).

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hNTH1 involved in homodimerization (Figs. 3–5) are apparently disordered (16); modification (truncation of first 55–80 residues) of this tail stimulated the activity of hNTH1 by itself (16). Apart from the classical allosteric regulation of enzyme catalysis by small molecules, protein–protein interaction distant from an active site have recently been shown to regulate catalysis and are regarded as molecular evolution of allosteric control (43). Our in vitro experiments, using proteins expressed in E. coli, also indicated that the homointeractions and formation of dimers involving the N-terminal tail might be a plausible allosteric effect for positive cooperativity of hNTH1 so as to enhance the rate of release of hNTH1 from its own product (Table II), and, hence, avoid product inhibition. A very recent all in vitro study with E. coli adenine DNA glycosylase (MutY) showed a dimeric mechanism for contextual target (substrate DNA) recognition. Unlike their pre-steady state enzyme kinetics, suggesting that the MutY utilizes the allosterically regulated dimerization event for target binding prior to initiating the catalytic steps, our results with hNTH1 demonstrated that the dimerization being independent of target binding regulates the turnover of the enzyme in an allosteric manner at the product release step (44). Various other proteins also have been shown to stimulate the activity of hNTH1. As is evident from Fig. 8, intriguingly these different factors, including the dimerization studied here, affect the unique kinetic step of the hNTH1 reaction to regulate its activity. XPG, which is essential for transcription-coupled repair of thymine glycol, a major substrate of hNTH1, and also involved in global BER for this lesion in vivo (45), has been reported to interact with hNTH1 (46) and stimulates its activity by increasing its binding to the substrate DNA (47). Thus XPG modulates the $k_1$ or $k_{-1}$ at the substrate-specific manner. In the case of Tg opposite adenine (A), APE1 affects the $k_6$ and/or $k_{-6}$ at the Schiff base resolution step by increasing dissociation of hNTH1 from the DNA N-glycosylase-generated AP:A site, resulting in abortion of AP lyase activity, and an increase in the turnover of N-glycosylase activity of hNTH1. Thus, in addition to $k_6$, $k_{-6}$ is also indirectly affected and enhanced by APE1 for the Tg:A substrate (42). Moreover, APE1 stimulates the rate of AP lyase activity ($k_p$) with Tg:G substrate without stimulating turnover or $k_{5}$, suggesting that hNTH1 remained tightly bound to its nicked AP site product after $\beta$-elimination (42). Earlier the Y box-binding protein 1 (YB-1), a damage-inducible transcription factor, also was shown to stimulate the activity of hNTH1 by inhibiting the Schiff base resolution step ($k_6$), thus enhancing the rates of both DNA N-glycosylase ($k_2$) and AP lyase ($k_3$) activities (23). Notably, all these studies with APE1 and YB-1 were conducted at subdimeric concentrations of hNTH1 (23, 42). Taken together, clearly, the delayed AP lyase activity ($k_3$), which leads to the emergence of $k_2$ and $k_{-2}$ for Schiff base resolution, and also the product inhibition or the rate of product release ($k_p$) inherent in hNTH1 activity are pivotal in the regulation of hNTH1 activity. Previously, we had documented that the N-terminal tail of hNTH1 inhibits the rate of product release ($k_5^*$; 16) and here we demonstrate that concentration-dependent dimerization of hNTH1 can affect the product release rate ($k_p$), thus stimulating the turnover of hNTH1. Therefore, XPG, YB-1, APE1, and dimerization regulate hNTH1 activity but each of these employs a unique mechanism. Obviously, in an in vivo situation different substrate lesions by virtue of their mutagenic or toxic characteristics and particular BER pathways, long or short patch and transcription-coupled, may have a significant impact on selecting the rate-limiting step for regulation of hNTH1 activity. Thus the in vivo significance of this paradigm remains to be elucidated.

Nonetheless, because very high intracellular concentrations (in $\mu$m range) of hNTH1 were found compared with those (in only nM range) required to observe in vitro dimerization, and because the hNTH1 dimer was detected in vivo even in the presence of other hNTH1-interacting proteins, XPG and YB-1 (both are present in HeLa cells; 48, 49), we suggest that dimerization plays a critical role in turnover of hNTH1 and regulation of its activity in the cell.

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