Excision of Products of Oxidative DNA Base Damage by Human NTH1 Protein

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ABSTRACT: A functional human homologue of *Escherichia coli* endonuclease III (Nth-Eco protein) has recently been cloned and characterized [Aspinwall, R., Rothwell, D. G., Roldan-Arjona, T., Anselmino, C., Ward, C. J., Cheadle, J. P., Sampson, J. R., Lindahl, T., Harris, P. C., and Hickson, I. D. (1997) *Proc. Natl. Acad. Sci. U.S.A., 94,* 109−114]. This enzyme, designated hNTH1 protein, shares an extensive sequence similarity with Nth-Eco protein and a related enzyme from *Schizosaccharomyces pombe* (Nth-Spo protein). We investigated the substrate specificity of this human enzyme for oxidative DNA base damage, using the technique of gas chromatography/isotope-dilution mass spectrometry. Four different DNA substrates damaged by various free radical-generating systems were used. 5-Hydroxycytosine, thymine glycol, 5-hydroxy-6-hydrothymine, 5,6-dihydroxycytosine, and 5-hydroxyuracil were substrates of hNTH1 protein among 17 lesions found in DNA substrates. The substrate specificity and excision kinetics of the human enzyme were found to be significantly different from those of Nth-Spo and Nth-Eco proteins.

Oxidative DNA base damage is mainly repaired by base-excision repair mechanisms (reviewed in ref *1*). DNA glycosylases are involved in the first step of this type of repair pathway and remove modified bases from the DNA strand (*1*). Recently, a human gene in chromosomal region 16p13.3 has been identified (*2*). This gene encodes a protein with a molecular mass of 34.3 kDa that possesses an extensive sequence similarity to *Escherichia coli* endonuclease III (Nth-Eco protein)[1] and a related enzyme from *Schizosaccharomyces pombe* (Nth-Spo protein) (*2*). This enzyme, designated hNTH1 protein, was overexpressed in *E. coli* and purified to apparent homogeneity. The hNTH1 protein was shown to exhibit DNA glycosylase activity on double-stranded DNA containing urea and thymine glycol residues as well as lyase activity (*2*). No activity of this enzyme for other products of oxidative DNA base damage has been demonstrated thus far.

We recently determined the substrate specificities of Nth-Eco and Nth-Spo proteins for a variety of lesions in oxidatively damaged DNA, and also measured excision kinetics of Nth-Spo protein using the technique of gas chromatography/isotope-dilution mass spectrometry (GC/IDMS) (*3, 4*). This technique is well suited for determination of substrate specificities and excision kinetics of DNA glycosylases because it can identify and quantify simultaneously a multitude of pyrimidine- and purine-derived lesions in DNA (*4*−*7*). In the present work, we report on the substrate specificity and excision kinetics of hNTH1 protein for base damage in DNA substrates that were damaged by various free radical-generating systems.

EXPERIMENTAL PROCEDURES

Materials.[2] Modified DNA bases, their stable isotope-labeled analogues and other materials for GC/MS were obtained as described (*8*). Irradiated or H_2O_2-treated DNA samples were prepared as described (*4, 5*). The purification of homogeneous hNTH1 protein has been described (*2*).

Enzymatic Assays. Aliquots of 100 μg of DNA samples were dried in a SpeedVac under vacuum. Samples were dissolved in the incubation mixture containing 50 mM phosphate buffer (pH 7.4), 100 mM KCl, 2 mM EDTA, and 2 mM dithiothreitol. The amount of hNTH1 protein added to the samples varied for each type of experiment. The total volume of the mixture was 110 μL. Three replicates of each mixture were incubated at 37 °C in a water bath with the active enzyme or inactivated enzyme or without the enzyme. The inactivation of the enzyme was achieved by heating it at 140 °C for 15 min. The measurement of excision kinetics was performed as described previously (*4*). The enzyme amount used was 2 μg per 100 μg of DNA in 110 μL of the incubation mixture, corresponding to an enzyme concentra-

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[1] Abbreviations: Nth-Eco protein, Nth protein of *Escherichia coli* (endonuclease III); Nth-Spo protein, Nth protein of *Schizosaccharomyces pombe*; hNTH1 protein, human Nth protein; GC/IDMS: gas chromatography/isotope-dilution mass spectrometry; Thy gly, thymine glycol; 5-OH-Cyt, 5-hydroxycytosine; 5-OH-6-HThy: 5-hydroxy-6-hydrothymine; 5,6-diOH-Cyt, 5,6-dihydroxycytosine; 5-OH-Ura, 5-hydroxyuracil.

[2] Certain commercial equipment or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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tion of 452 nM. Three replicates of DNA samples were incubated with (2 μg) or without hNTH1 protein at 37 °C for 30 min. After incubation, 270 μL of cold ethanol were added to DNA samples. The samples were kept at −20 °C for 2 h, and centrifuged at 10000 rpm for 30 min at 4 °C. DNA pellets and supernatant fractions were separated.

Analysis by GC/IDMS. Aliquots of stable isotope-labeled analogues of modified DNA bases were added as internal standards to DNA pellets and to supernatant fractions. Pellets were dried under vacuum in a SpeedVac, hydrolyzed, and subsequently lyophilized as described (*4*). After removal of ethanol in a SpeedVac, supernatant fractions were lyophilized for 18 h without prior hydrolysis. Both supernatant fractions and hydrolysates of pellets were derivatized and then separately analyzed by GC/IDMS with selected-ion monitoring as described (*9*).

RESULTS

The hNTH1 protein has been previously demonstrated to have a DNA glycosylase activity on double-stranded polydeoxynucleotides containing urea and thymine glycol (Thy gly) residues (*2*). The purpose of the present work was to investigate whether this enzyme possesses any other activity for modified DNA bases in oxidatively damaged DNA containing a multitude of base lesions. We used four DNA substrates that were prepared by treatment with four different free radical-generating systems, namely γ-irradiation under N_2O or air, and H_2O_2 in the presence of Fe(III)-EDTA or Cu(II). Of the 17 lesions identified in these DNA substrates (*4*), 5-hydroxycytosine (5-OH-Cyt), Thy gly, 5-hydroxy-6-hydrothymine (5-OH-6-HThy), 5,6-dihydroxycytosine (5,6-diOH-Cyt), and 5-hydroxyuracil (5-OH-Ura) were efficiently excised by hNTH1 protein. 5-OH-6-HThy was identified in DNA γ-irradiated under N_2O only, because oxygen inhibits the formation of this compound (*10, 11*). The structures of these lesions are given elsewhere (*4*). Other lesions were not significantly excised. The levels of five substrate lesions in DNA samples were as reported previously (*4*). These levels were significantly reduced in DNA pellets incubated with the active hNTH1 protein when compared with those in DNA pellets treated with the inactivated enzyme or without the enzyme. The amounts found in the supernatant fractions of DNA substrates incubated with the active enzyme were similar to those removed from pellets, unequivocally proving excision of these lesions (data not shown).

Excision of 5-OH-Cyt, Thy gly, 5-OH-6-HThy, 5,6-diOH-Cyt, and 5-OH-Ura increased as a function of the enzyme concentration and incubation time. The enzyme amounts tested varied from 0.5 μg to 4 μg per 110 μL of the incubation mixture and the time varied from 5 min to 60 min. At enzyme amounts > 4 μg of the enzyme, no further excision was observed (data not shown). Figure 1 illustrates excision of 5-OH-Cyt and Thy gly as a function of incubation time using 2 μg of the enzyme. Kinetics of excision were determined by measurement of excision at different substrate concentrations. The concentration ranges of the lesions were as previously reported (*4*). Excision of these lesions followed Michaelis−Menten kinetics (*12*). Kinetic constants were obtained by utilizing Lineweaver−Burk plots (*12*). Figure 2 illustrates representative plots for excision of 5-OH-Cyt from various DNA substrates. Tables 1−3 show the kinetic

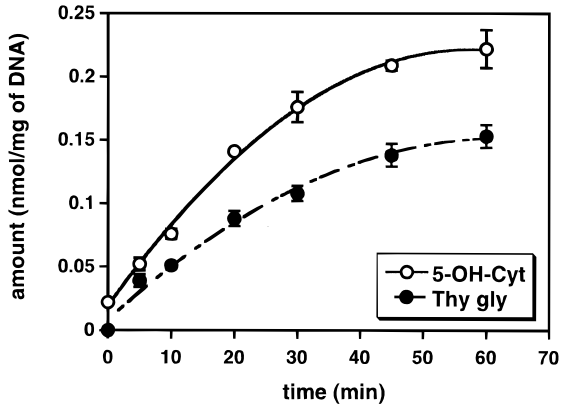

FIGURE 1: Excision of 5-OH-Cyt and Thy gly by hNTH1 protein as a function of incubation time. DNA γ-irradiated under N_2O was used. The amount of the enzyme was 2 μg per 100 μg of DNA. The amounts given on the *y*-axis represent those found in the supernatant fractions. One nanomole of a lesion corresponds to ≈32 lesions/10^5 DNA bases.

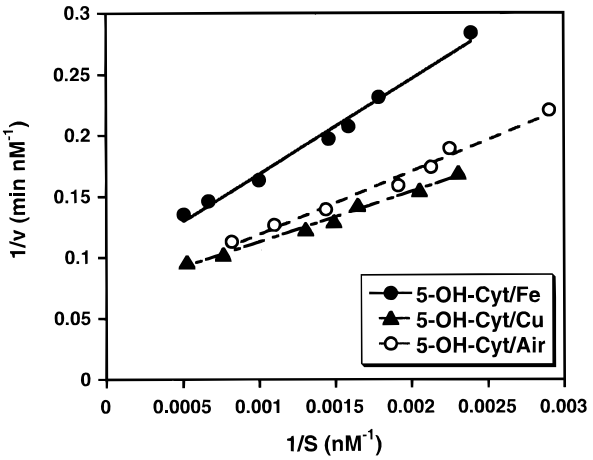

FIGURE 2: Lineweaver−Burk plots for excision of 5-OH-Cyt by hNTH1 protein from various DNA substrates. Enzyme amount was 2 μg per 100 μg of DNA. The amounts given on the *y*-axis represent those found in the supernatant fractions. Air: DNA irradiated under air; Fe: DNA treated with H_2O_2/Fe(III)-EDTA; Cu: DNA treated with H_2O_2/Cu(II).

constants and standard deviations (n = 7), which were obtained by a linear least-squares analysis of the data. The kinetic constants for excision of 5,6-diOH-Cyt could not be reliably determined in the case of γ-irradiated and H_2O_2/Fe(III)-EDTA-treated DNA substrates because of low excision at lower concentrations. For the same reason, neither could the kinetic constants be determined for excision of 5-OH-Ura in the case of DNA γ-irradiated under N_2O.

The k_{cat} values for excision of 5-OH-Cyt and Thy gly from DNA γ-irradiated under N_2O were similar. Both were significantly greater than that of 5-OH-6-HThy (Table 1). In contrast, the k_{cat} values of Thy gly were twice as high as those of 5-OH-Cyt in the case of the other DNA substrates. The K_M values of Thy gly were significantly greater than those of 5-OH-Cyt, 5-OH-6-HThy, 5,6-diOH-Cyt, and 5-OH-Ura in all cases (Table 2). The specificity constants (k_{cat}/K_M values) for excision of 5-OH-Cyt were significantly greater than those of Thy gly, 5,6-diOH-Cyt, and 5-OH-Ura for all DNA substrates, indicating a preference of hNTH1 protein for excision of 5-OH-Cyt (Table 3). On the other hand, the enzyme had a similar preference for excision of 5-OH-Cyt

Table 1: Catalytic Constants [$k_{cat} \times 10^3$ (min^{-1})[a]] for Excision of Pyrimidine Lesions from DNA by hNTH1 Protein

DNA substrate	5-OH-Cyt	Thy gly	5-OH-6-HThy	5,6-diOH-Cyt	5-OH-Ura
irradiation/N_2O	19.3 ± 3.0^{f}	$17.3 \pm 3.2^{b,c,d,f}$	10.1 ± 0.7	–	–
irradiation/air	$28.0 \pm 3.4^{e,h}$	56.4 ± 9.7^{h}	–	–	10.0 ± 2.6
H_2O_2/Fe-EDTA	$20.9 \pm 1.6^{e,h}$	45.1 ± 4.7^{h}	–	–	6.0 ± 1.3^{d}
H_2O_2/Cu	$26.4 \pm 1.3^{e,g}$	49.5 ± 4.0^{g}	–	13.0 ± 5.8	19.1 ± 5.13

[a] Values represent the mean ± standard deviation ($n = 7$). ($k_{cat} = V_{max}$/[enzyme]) ([enzyme] = 452 nM). [b] Statistically different from the value in line 2 ($P < 0.05$). [c] Statistically different from the value in line 3 ($P < 0.05$). [d] Statistically different from the value in line 4 ($P < 0.05$). [e] Statistically different from the value in column 2 ($P < 0.05$). [f] Statistically different from the value in column 3 ($P < 0.05$). [g] Statistically different from the value in column 4 ($P < 0.05$). [h] Statistically different from the value in column 5 ($P < 0.05$).

Table 2: Michaelis Constants [K_M (nM)[a]] for Excision of Pyrimidine Lesions from DNA by hNTH1 Protein

DNA substrate	5-OH-Cyt	Thy gly	5-OH-6-HThy	5,6-diOH-Cyt	5-OH-Ura
irradiation/N_2O	$548 \pm 91^{c,e}$	$1101 \pm 211^{b,c,d,f}$	371 ± 45	–	–
irradiation/air	764 ± 96^{e}	$3864 \pm 587^{d,h}$	–	–	635 ± 179
H_2O_2/Fe-EDTA	$861 \pm 73^{d,e}$	$3438 \pm 330^{d,h}$	–	–	579 ± 123
H_2O_2/Cu	576 ± 37^{e}	1920 ± 163^{g}	–	718 ± 282	1093 ± 260

[a] Values represent the mean ± standard deviation ($n = 7$). [b] Statistically different from the value in line 2 ($P < 0.05$). [c] Statistically different from the value in line 3 ($P < 0.05$). [d] Statistically different from the value in line 4 ($P < 0.05$). [e] Statistically different from the value in column 2 ($P < 0.05$). [f] Statistically different from the value in column 3 ($P < 0.05$). [g] Statistically different from the value in column 4 ($P < 0.05$). [h] Statistically different from the value in column 5 ($P < 0.05$).

Table 3: Specificity Constants [$k_{cat}/K_M \times 10^5$ (min^{-1}·nM^{-1})[a]] for Excision of Pyrimidine Lesions from DNA by hNTH1 Protein

DNA substrate	5-OH-Cyt	Thy gly	5-OH-6-HThy	5,6-diOH-Cyt	5-OH-Ura
irradiation/N_2O	$3.52 \pm 0.56^{c,e}$	$1.57 \pm 0.30^{d,f}$	2.73 ± 0.21	–	–
irradiation/air	$3.67 \pm 0.44^{c,e,h}$	1.46 ± 0.25^{d}	–	–	1.58 ± 0.42
H_2O_2/Fe-EDTA	$2.42 \pm 0.19^{d,e,h}$	1.31 ± 0.14^{d}	–	–	1.04 ± 0.22
H_2O_2/Cu	$4.58 \pm 0.23^{e,g}$	2.58 ± 0.21	–	1.81 ± 0.82	1.74 ± 0.47

[a] Values represent the mean ± standard deviation ($n = 7$). [b] Statistically different from the value in line 2 ($P < 0.05$). [c] Statistically different from the value in line 3 ($P < 0.05$). [d] Statistically different from the value in line 4 ($P < 0.05$). [e] Statistically different from the value in column 2 ($P < 0.05$). [f] Statistically different from the value in column 3 ($P < 0.05$). [g] Statistically different from the value in column 4 ($P < 0.05$). [h] Statistically different from the value in column 5 ($P < 0.05$).

and 5-OH-6-HThy from DNA γ-irradiated under N_2O. Differences between DNA substrates were also noted (Tables 1−3).

DISCUSSION

The results show the ability of hNTH1 protein to excise five pyrimidine-derived lesions from DNA. Four different DNA substrates were used for this study. These contained a multitude of modified DNA bases with varying amounts. The results indicate that the substrate specificity of hNTH1 protein is similar to that of Nth-Spo protein (*4*). Similar DNA substrates were used for both studies. This enabled us to compare excision kinetics of both enzymes under similar conditions. Kinetic parameters of these enzymes were similar in some cases and significantly different in others. The k_{cat} values of 5-OH-Cyt excision from irradiated samples were similar in both cases (ref *4* and this work), but significantly higher for excision from H_2O_2-treated samples in the case of Nth-Spo protein (*4*). Thy gly had similar values in the case of DNA irradiated under air and DNA treated with H_2O_2/Fe-EDTA. However, k_{cat} was significantly higher for other DNA substrates in the case of Nth-Spo protein. 5-OH-6-HThy also had a significantly greater k_{cat} value for excision by Nth-Spo protein than that by hNTH1 protein. 5,6-diOH-Cyt and 5-OH-Ura had similar values for both enzymes where measurements of excision kinetics of these compounds could be done. The K_M values had a similar pattern and significant differences between the two enzymes were noted. The specificity constants (k_{cat}/K_M) of 5-OH-Cyt excision were greater than those for Thy gly excision in all cases of DNA substrates for hNTH1 protein, indicating 5-OH-Cyt as the main substrate for this enzyme. In the case of Nth-Spo protein, however, k_{cat}/K_M values for both 5-OH-Cyt and Thy gly were similar except for DNA irradiated under N_2O, where 5-OH-Cyt was the preferred substrate (*4*). The specificity constants of 5-OH-Cyt excision for both enzymes were quite similar. This was also true for Thy gly, 5,6-diOH-Cyt, and 5-OH-Ura (ref *4* and this work). Taken together, the results show that hNTH1 and Nth-Spo proteins have common substrates. For most base products, however, the excision kinetics of these enzymes significantly differ from one another. The substrate specificities of hNTH1 and Nth-Spo proteins are somewhat different from that of Nth-Eco protein, as the latter recognizes additional lesions (*3*). A comparison of the substrate lesions of Nth-Eco and Nth-Spo proteins can be found in a recent paper (*4*). The narrower substrate specificities of both Nth-Eco and Nth-Spo proteins may suggest that eukaryotic cells possess additional DNA glycosylases yet to be discovered. Such DNA glycosylases may recognize lesions that are handled by Nth-Eco protein and endonuclease VIII in *E. coli*. This notion is supported by recent findings that *Saccharomyces cerevisiae* Ntg1 and Ntg2 proteins recognize and excise a variety of DNA base products from damaged DNA (*13*, *14*).

It is interesting to note that the K_M values for hNTH1 and Nth-Spo proteins are higher than those for Nth-Eco protein reported for a limited number of products (*15*). Although this difference may be related mainly to the specific assay

and different DNA substrates used in each case, it may also reflect different kinetic properties of the enzymes. In the same context, it should not be ruled out that the overexpression and purification system used for the two eukaryotic proteins may affect their enzymatic properties. Furthermore, DNA substrates with multiple base lesions as used in this work may give rise to high K_M values because the enzyme acts on several substrates simultaneously. Each modified base may act as a competitor to other lesions for excision; thus it may act as an inhibitor with respect to others. As a result, the kinetics of the lesions for excision from DNA substrates with multiple lesions may be different from those obtained with oligonucleotides or DNA having a single lesion (*15*−*18*). High K_M values observed in this work are in agreement with those obtained under similar conditions with other DNA glycosylases such as *E. coli* Fpg protein, *Drosophila* ribosomal S3 protein, and *S. cerevisiae* Ogg1, Ntg1, and Ntg2 proteins (*5*−*7*, *14*). It should be pointed out that the excision kinetics of Nth-Eco protein under the conditions similar to those in this work have not been measured. Therefore, a comparison of its excision kinetics with those of hNTH1 and Nth-Spo proteins is not possible at present time.

Kinetics of excision of the lesions by hNTH1 protein varied among DNA substrates. This was more emphasized for Thy gly than for 5-OH-Cyt. The latter compound had similar k_{cat} values in all four cases and similar K_M and k_{cat}/K_M values in three of the four DNA substrates. The exception was H_2O_2/Fe-EDTA-treated DNA, for which 5-OH-Cyt was the least preferred substrate. The k_{cat}/K_M value for excision of Thy gly from H_2O_2/Cu-treated DNA was highest. Differences between the kinetic constants for excision of 5-OH-Ura from DNA irradiated under air and H_2O_2/Fe-EDTA-treated DNA were also noted. The dependence of excision kinetics of hNTH1 protein on the nature of DNA substrate is in good agreement with previous results obtained with other DNA glycosylases (*4*, *5*, *7*). Possible explanations for this phenomenon may include the notion that damage to DNA under different conditions may generate a different, sequence-dependent distribution of modified bases. The formation of the lesions and their enzymatic recognition may depend on the sequence. For example, Cu ions are known to form a complex with guanine in DNA (*19*). For this reason, Cu(II)-dependent damage may be site-specific. In the same context, multiple damaged sites in DNA, as generated by ionizing radiation (*20*, *21*), have been found to interact with the action of DNA glycosylases such as Nth-Eco protein and *E. coli* endonuclease VIII (*22*). The evidence showed that the excision of pyrimidine lesions by these enzymes strongly depended on the position and type of lesions on the opposite strand (*22*). Also known is that closely spaced lesions on opposite DNA strands may affect the action of DNA repair enzymes (*23*, *24*).

In conclusion, the hNTH1 protein is specific for several pyrimidine lesions in DNA damaged by various free radical-generating systems. Excision kinetics of the lesions differed from one another for a given DNA substrate. Excision kinetics of each modified base also varied among four different DNA substrates, in agreement with previous results on other DNA glycosylases. The substrate specificity of this enzyme is similar to that of Nth-Spo protein, although excision kinetics substantially differ in most cases.

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